

Angiogenesis and Epithelial Ovarian Cancer:

**A Study of Vascular Endothelial
Growth Factor (VEGF) and
associated factors in the
pathogenesis and response to
therapy of Epithelial Ovarian
Cancer.**

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DECLARATION

I, Ghazna Siddiqui, confirm that all work presented in this thesis is my own. Where information has been derived from other sources, I can confirm that this has been indicated in the thesis.

DEDICATIONS

I would like to dedicate this thesis to my mother, who has taught me that the best accomplishment is the search for truth and knowledge, and that the search for knowledge is the true component of Being.

I would also like to dedicate this thesis to my husband, Karim Elmasry, without whose support this thesis would not have been possible. He has offered both technical and emotional support and remained patient and encouraging throughout the writing of this thesis.

ABSTRACT

In this thesis, I aim to explore the role of angiogenesis in epithelial ovarian cancer (EOC) and its correlation with risk of disease development, clinical outcome and disease response to platinum based chemotherapy.

Immunohistochemical (IHC) techniques were used to examine the surgical specimens of 105 patients with primary EOC, FIGO stages I to IV. The results of vascular endothelial growth factor (VEGF) expression were correlated with clinicopathological variables and overall patient survival. No correlation between VEGF expression and clinicopathological factors was identified. However VEGF expression was found to significantly correlate with overall patient survival and a prognostic factor independent of the stage of the disease and residual tumour status ($p < 0.0001$).

The association between VEGF-A expression in the tumour and the patient response to platinum based chemotherapy was examined by studying the expression of VEGF-A in 66 patients with advanced stage EOC (FIGO stages III-IV). Expression of the protein was correlated with platinum sensitivity and overall patient survival. I demonstrated that platinum resistant EOC was associated with a higher proportion of high VEGF expression in the tumour.

To examine whether the differential production of VEGF is associated with the risk of EOC, I conducted a case control study to investigate whether the polymorphism in the VEGF gene – VEGF 1154 A/G genotype, which is known to be associated with the increased production of VEGF protein, is associated with the risk of EOC. There was a statistically significant difference in the distribution of the genotype of the VEGF 1154 A/G marker in the cases as compared with the controls. For the high-risk (GG) genotype, the difference between the patient and the control groups was statistically significant.

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Chapter 1: INTRODUCTION

1.1 GENERAL

Ovarian cancer is the fourth most common cancer after lung, breast and colon among women, and the leading cause of death from a gynaecological malignancy. As symptoms preceding the development of ovarian cancer are vague and insidious 70% of patients present at an advanced stage, i.e. International Federation of Gynaecology and Obstetrics (FIGO) stages III and IV. Despite advances in surgery and chemotherapy the death rate has changed little over the last twenty five years.

The molecular biology underlying epithelial ovarian cancer (EOC) remains poorly understood. Tumour angiogenesis is critical for tumour growth beyond 2mm³ and is associated with prognostic significance in a variety of solid malignancies, including ovarian cancer.

In this thesis, I aim to explore the role of angiogenesis in epithelial ovarian cancer and its correlation with risk of disease development, clinical outcome and disease response to platinum based chemotherapy.

Immunohistochemical (IHC) techniques were used to examine the surgical specimens of 105 patients with primary epithelial ovarian cancer, FIGO stages I to IV. The results of vascular endothelial growth factor (VEGF) expression were correlated with clinicopathological variables and overall patient survival. No correlation between VEGF expression and clinicopathological factors was identified. However VEGF expression was found to significantly correlate with overall patient survival and a prognostic factor independent of the stage of the disease and residual tumour status ($p < 0.0001$).

To further the understanding of the role of angiogenesis and disease response in EOC, the association between VEGF-A expression in the tumour and the patient response to platinum based chemotherapy was examined by studying the immunohistochemical expression of VEGF-A in 66 patients with

advanced stage EOC (FIGO stages III-IV). Expression of the protein was correlated with platinum sensitivity and overall patient survival. In this study I demonstrated that platinum resistant EOC was associated with a higher proportion of high VEGF expression in the tumour; 81% of these patients had a high VEGF score as compared to only 29% high VEGF expression in the group of patients that were sensitive to platinum based chemotherapy. I also illustrated that overall median survival was 10.79 months (SD7.75) in the patients with a high VEGF intra-tumour VEGF score versus 36.36 months (SD21.69) in patients with a low VEGF score in the tumour. VEGF expression was found to be significantly correlated with patient survival ($p < 0.0001$).

To evaluate the role of angiogenesis and the development on EOC, and to examine whether the differential production of VEGF is associated with the risk of ovarian cancer, I conducted a case control study to investigate whether the polymorphism in the VEGF gene – VEGF 1154 A/G genotype, which is known to be associated with the increased production of VEGF protein, is associated with the risk of epithelial ovarian cancer. In this case control study I recruited 296 epithelial ovarian cases and 298 controls. The cases were recruited from 3 tertiary centres in Pakistan, in the cities of Karachi and Lahore. The controls were hospital matched controls selected from outpatient clinics, with no personal history of cancer. All study subjects were of Pakistani ancestry. Genotyping was performed using standard methods. STATA was used for statistical analysis and procedures such as logistic regression, chi square and ANOVA were used for analysis. I found that there was a statistically significant difference in the distribution of the genotype of the VEGF 1154 A/G marker in the cases as compared with the controls. For the high-risk (GG) genotype, the difference between the patient and the control groups was statistically significant (OR 0.43, 95% CI = 0.19-0.95, $p=0.37$). 39% of the cases were found to be homozygous for the GG genotype, compared to only 16% of the control subjects. Our results illustrated a significant difference between the cases and controls in allele frequencies ($p < 0.042$) and carriage of the 1154 G allele was associated with an increased risk of ovarian cancer. This result may be explained, and is biologically plausible, as the VEGF 1154 G/G polymorphism is known to be associated

with increased VEGF production. Validation of the effect of this SNP is required by larger sample sizes.

1.2 ANGIOGENESIS

“One is almost forced to the conclusion that there is, associated with the viable growing tumor, some blood vessel growth stimulating factor.” G. Ide et al, Am J Roentgenol 1939;42:891-9.

The functioning of cells in humans requires a highly dependable supply of oxygen and nutrients. Given that the diffusion of oxygen in tissues is limited to 100-200µm, a highly developed vascular system has evolved to ensure that all cells are within this distance of an oxygen supply. Blood vessels are essentially composed of endothelial cells. These interconnect to form tubes that direct blood flow and maintain tissue perfusion. In higher organisms, the formation of blood vessels is through two distinct processes: vasculogenesis and angiogenesis. In *vasculogenesis* blood vessels are derived from mesoderm by the differentiation of angioblasts. These angioblasts are endothelial cells that have not formed a lumen and contribute to the formation of primitive vascular networks. This process predominantly takes place in the developing embryo through a process that involves endothelial cell division, selective degradation of the basement membrane and the surrounding extracellular matrix, endothelial cell migration and the formation of a tubular structure. The endothelial cells then undergo tissue-specific changes to generate structurally distinct vessels. *Angiogenesis*, on the other hand is the formation of vessels from pre-existing vasculature through processes such as sprouting, pruning and intussusception. However, the differentiation between these two processes, is often not a sharp one, and the term ‘angiogenesis’ is used to summarise all different types and modifications of arterial vessel growth.

The process of angiogenesis involves the migration of endothelial cells from the parent vessel towards a chemo-attractant. As well as the ability to promote endothelial cell migration, angiogenic factors must also degrade the extracellular matrix through which the cells move. Endothelial cell invasion as part of the angiogenic process involves secretion of urokinase-type plasminogen activator and its inhibitor (PAI-1). Fine maintenance of the proteolytic balance seems to be crucial for the correct development of new blood vessels ¹. Having migrated, the endothelial cells must proliferate, lay down their basement membrane and form a lumen, which is the basis of the new capillary. This sequence of events (endothelial cell proliferation, migration, basement membrane degradation and new lumen organisation) is the basis of angiogenesis. The molecular mechanisms that regulate these individual processes are subject to a complex control system with pro and anti-angiogenic factors.

Angiogenesis can be a physiological process and occurs in the highly ordered process in the female reproductive cycles (ovulation, menstruation, pregnancy) and wound healing. This process is usually focal and self-limited. However angiogenesis can also be a pathological process ². The concept of angiogenesis dependent diseases originated in 1972 with the recognition that certain non-neoplastic conditions such as chronic inflammatory psoriasis depend on persistent neovascularisation to provide a channel for the continuing delivery of inflammatory cells to the inflammatory site ³⁻⁵. Following on from this, other non-neoplastic conditions were recognised to be angiogenesis dependent, including autoimmune disease, age related macular degeneration, atherosclerosis ^{1 3 6}, peptic ulcers ⁷ and atherosclerosis ⁸.

DISEASE	SYMPTOMS
Diabetic Neuropathy	Loss of vision
Rheumatoid Arthritis	Pain and immobility
Atherosclerotic Plaques	Chest pain, dyspnoea
Crohn's Disease	Intestinal Bleeding
Psoriasis	Persistent severe itching
Uterine fibroids	Vaginal bleeding, abdominal pain
Cancer	Bleeding, thrombosis, anaemia, abdominal ascites; bone pain.

Table 1.1. Role of angiogenesis in pathological processes.

Adapted from Angiogenesis in Cancer and other diseases in Nature Sept 2000
Carmeliet P 407, 249-257

1.2.1 The Angiogenic Switch

Angiogenesis is controlled and regulated by a range of pro-angiogenic and anti-angiogenic factors (Figure 1.1)². Several years ago, Hanahan and Folkman postulated the angiogenic switch hypothesis⁹. The “angiogenic switch” is a term that refers to the balance between pro- and anti-angiogenic factors. When the balance is in favour of the pro-angiogenic factors, the balance is tipped favouring angiogenesis. More than 20 stimulators and inhibitors have so far been described. The positive factors include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin and angiopoietin amongst many. These can be exported from the tumour cell, mobilized from the extracellular matrix or released by macrophages attracted to the tumour. Thus, angiogenesis is an interplay of positive and negative regulation of several factors.

Stimulators of Angiogenesis
Vascular endothelial growth factor
PDECGF/TP
Angiopoietin1 and 2
Insulin like growth factor
Epidermal growth factor
Placental growth factor
Tumour necrosis factor
Matrix metalloproteinases
Ephrin family
Transforming growth actor
Granulocyte macrophage stimulating factor
Interleukin 8

Inhibitors of Angiogenesis
Vascular endothelial growth factor inhibitor
Thrombospondin 1
Angiostatin
Endostatin
Vasostatin
Interleukin 1, 2
Restin
Antithrombin 3
Meth 1 and Meth 2
Interferon a and i
Interferon 6



Figure 1.1: The angiogenic switch: a balance of pro and anti-angiogenic factors

One of the most widely studied angiogenic activators is vascular endothelial growth factor (VEGF). VEGF is widely distributed and has been shown to play a coordinated role in endothelial cell proliferation and assembly of the vessel wall in a variety of normal and abnormal circumstances. Members of the VEGF family together with members of another pro-angiogenic factor, angiopoietin, and at least one member of the Ephrin family all work in a complementary and coordinated manner to form functional vessels. In addition many other growth factors that are not vascular endothelium specific are also required for blood vessel formation, such as members of the platelet derived growth factors and transforming growth factors (TGF). These factors also have crucial roles in many other systems.

1.2.2 Vascular Endothelial Growth Factor (VEGF)

1.2.2.1 The VEGF ligand

Vascular endothelial growth factor is a heparin binding glycoprotein, and was originally identified in the media of conditioned normal bovine pituitary follicostellate cells ¹⁰ and by a variety of transformed cell lines ¹¹. It is a multifunctional cytokine with potent angiogenic activity. VEGF is a potent and specific mitogen for vascular endothelial cells ¹²⁻¹⁴. It stimulates the full cascade of events required for angiogenesis both in vitro and in vivo ^{14 15} and it greatly augments the permeability of existing microvasculature. It is a potent multifunctional cytokine that has several potentially independent effects on the vascular endothelium including:

- 1) Endothelial mitogenesis
- 2) Mitogenesis
- 3) Vascular tone
- 4) The production of vasoactive molecules.
- 5) Stimulation of monocytes chemotaxis^{16 17}

VEGF also functions as a potent pro-survival (anti-apoptotic) factor for endothelial cells in newly formed vessels. VEGF is a family of growth factors belonging to the platelet derived growth factor/VEGF supergene family, having a homodimer structure with 8 conserved cysteine residues ^{18 19}. Figure 1. 2 represents the three dimensional structure of VEGF.

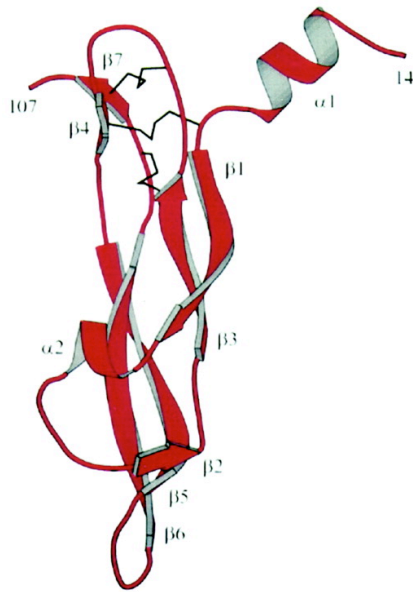


Figure 1.2. Ribbon representation of the receptor-binding domain of VEGF.

The VEGF family has at least 7 members: VEGF A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PlGF) ^{20 21}. All members of the family are encoded in the mammalian genome except for VEGF-E and VEGF-F, these having been discovered encoded by viruses and in the venom of some snakes respectively. The human VEGF gene is mapped to chromosome 6p21.3. All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (VEGFRs) on the cell surface causing them to dimerise and become trans-phosphorylated.

The best characterised of the VEGF family members is VEGF-A, a key player in vasculogenesis, the formation of blood vessels from progenitor cells, as well as angiogenesis.

VEGF-A, a 34 to 42 kDa dimeric disulphide-bound glycoprotein, exists in at least 7 homodimeric isoforms. The monomers consist of 121, 145, 148, 165, 183, 189 and 206 amino acids and are generated by alternative splicing ²². The major subtypes of VEGF-A in humans are the 121,165, and 189-amino acid type. The variation in size suggests different roles. VEGF 121 and 165 are secreted in soluble forms, whereas VEGF 145, 189, and 206 remain associated with plasma cell membrane surface because of their interaction with proteoglycans. VEGF-A₁₂₁ does not contain a basic stretch, whereas VEGF-A₁₆₅ and VEGF-A₁₈₉ carry basic stretches in the carboxyl terminal

region. VEGF-A₁₆₅ is the dominant subtype among VEGF-A proteins in terms of amount and biological activity. Via its basic stretch, VEGF-A₁₆₅ modestly binds with neuropilin-1 (Nrp1) and heparin, which contributes to a gradient of VEGF-A₁₆₅ surrounding VEGF-A-expressing cells. This gradient is known to be important for the stimulation of vascular endothelial cells and for strong angiogenesis²³. VEGF-A is one amino-acid shorter in mice. Mice carrying only the 164-allele (VEGF-A_{164/164} mice) are viable but the other two genotypes (VEGF-A_{120/120}, and VEGF-A_{188/188} mice) are embryonic lethal, indicating that VEGF-A₁₆₄ is essential and sufficient for normal development of blood vessels in mice²⁴.

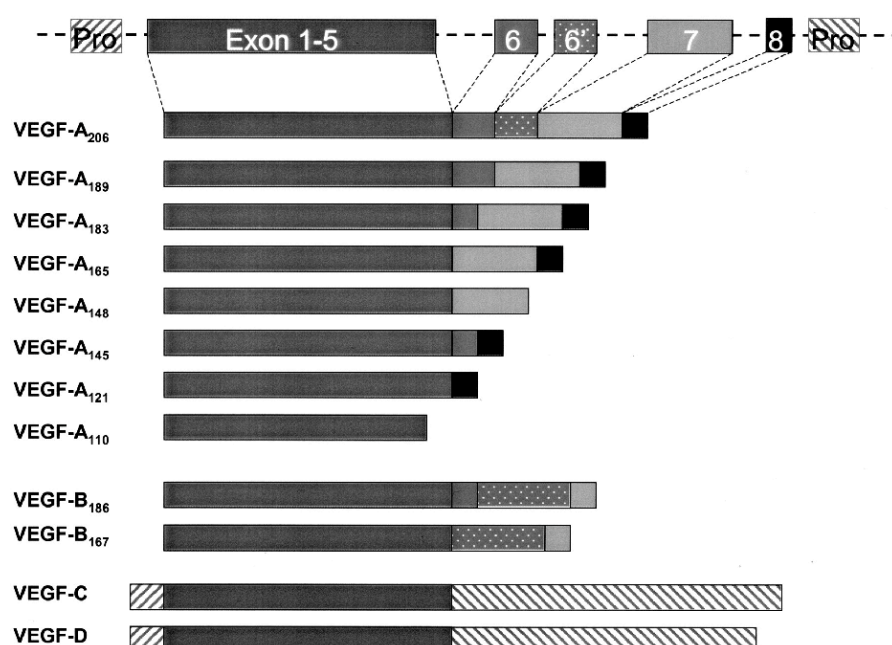


Figure 1.3. Gene structure of VEGF-A, VEGF-B, VEGF-C, and VEGF-D²⁵.

The VEGF-A gene consists of eight exons that give rise to seven isoforms of 121, 145, 148, 165, 183, 189, and 206 amino acids through differential splicing. An additional VEGF-A isoform of 110 amino acids results from proteolytic cleavage. VEGF-B exists as two isoforms of 167 and 186 amino acids. VEGF-C and VEGF-D are proteolytically released from their respective proproteins. All VEGF family members share a highly preserved VEGF homology domain, encoded by exons 1 to 5.

The expression of the VEGF-A gene is up-regulated via hypoxia^{26 27}, estrogen, NF- κ B pathways, and others. Under hypoxic conditions, HIF α proteins (HIF1 α and HIF2 α) are stabilised due to a block of ubiquitin-dependent degradation. Since the VHL (von Hippel-Lindau) protein plays a major role in the degradation of HIF α , renal cell carcinoma patients carrying a VHL-gene-deficiency generally show high levels of VEGF-A mRNA and protein in tumour tissues, and strong tumour angiogenesis.

VEGF-A binds and activates VEGFR1 and VEGFR2 but not VEGFR3 (see below figure 4).

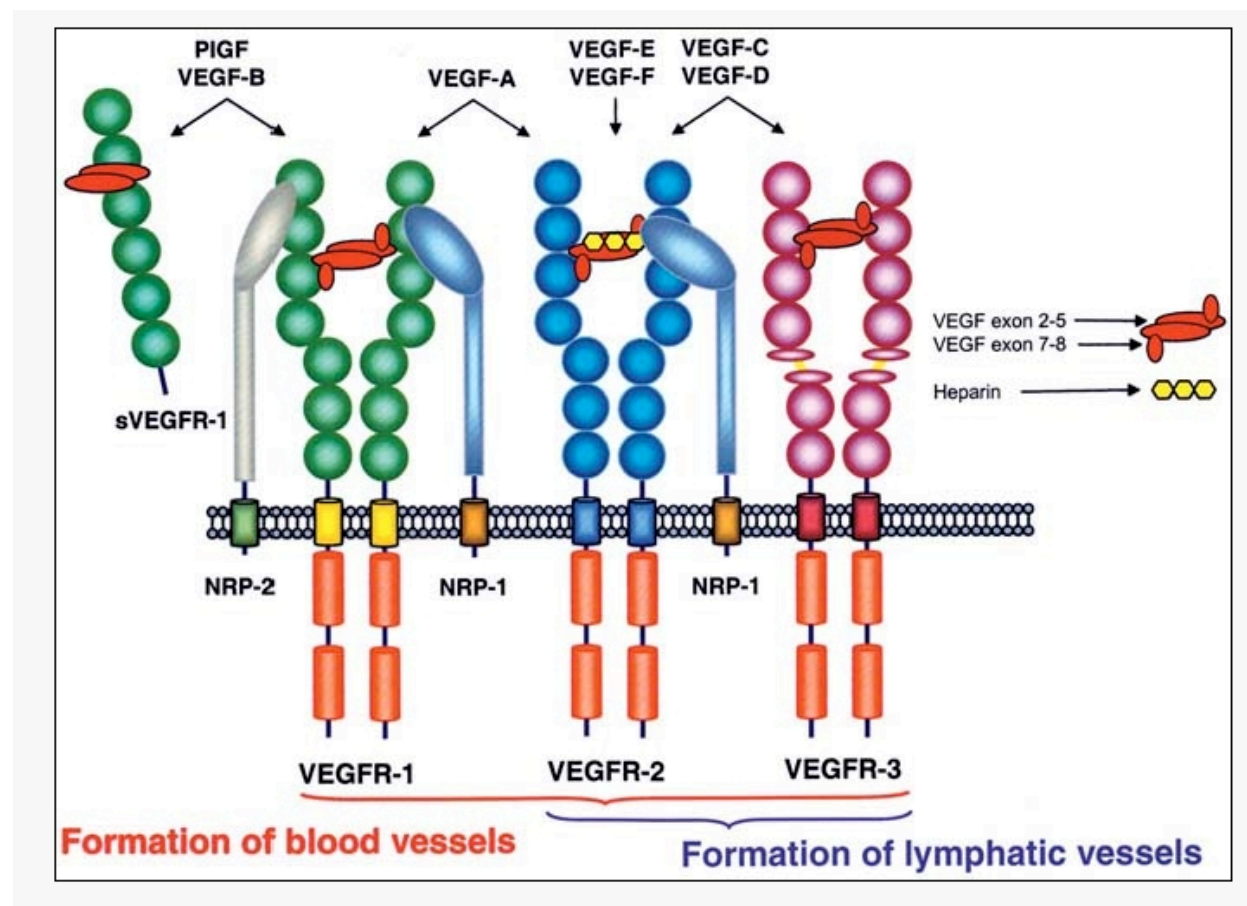


Figure 1.4. Schematic representation of VEGF family ligands and their receptors¹⁸.

In contrast to VEGF-A, VEGF-B plays a less pronounced role in the vascular system appearing to only to play in role in the maintenance of newly formed

blood vessels during pathological conditions ²⁸. High expression of VEGF-B is seen in adult myocardium, skeletal muscle and pancreas. Alternative splicing of VEGF-B, discovered in 1995, generates 2 isoforms of 167 and 186 amino acids. The 2 isoforms are differentially expressed, VEGF-B₁₆₇ being dominant, suggesting that splicing events are strictly regulated. VEGF-B is abundantly expressed in the adult myocardium, skeletal muscle and pancreas. VEGF-B exerts its effects via VEGFR1 ^{29 30}.

VEGF-C and VEGF-D, crucial regulators of lymphangiogenesis ³¹, are originally synthesized in premature forms, and processed by digestion with furin and other proprotein convertases to shorter forms ³². After processing, they acquire a strong ability to bind VEGFR3, and induce lymphangiogenesis. The VEGF-C gene starts to be expressed early in embryogenesis, and plays an essential role in the development of lymphatic endothelial cells from veins at embryonic E9.5-10.5 in mice. Not only homozygotes but also heterozygotes for the VEGF-C allele (VEGF-C^{+/-} mice) often die in the perinatal stage due to a dysfunction of lymph vessels, severe systemic oedema and the accumulation of lipids in body fluids. This indicates that half the normal level of VEGF-C protein is insufficient for the absorption of tissue fluids and lipids. VEGF-C or VEGF-D-overexpressing tumours are lymphangiogenic and highly metastatic to lymph nodes. Thus, these ligands are important targets for suppression of lymph-node metastasis ³³.

1.2.2.2 The vascular endothelial growth factor receptor (VEGFR)

The biological effects of VEGF are mediated through the activation of specific tyrosine kinase receptors expressed mainly on angioblast and endothelial cells ³⁴. The VEGFR family consists of three members: VEGFR1, VEGFR2 and VEGFR3. The VEGF receptors have an extracellular portion consisting of immunoglobulin-like domains, a single transmembrane spanning region and an intracellular portion containing a split tyrosine-kinase domain ¹⁹. VEGFR 1 and 2 bind VEGF A and play a central role in the regulation of angiogenesis:

whereas VEGFR3 tightly binds VEGF C and D and stimulates lymphangiogenesis.

VEGFR1 (fms-like tyrosine kinase Flt-1) has a very high affinity for VEGF-A at $K_d=2$ to 10 pM. Despite the high affinity, its tyrosine kinase activity is weak (about 10-fold lower than that of VEGFR2). Based on this weak activity, under physiological conditions, VEGFR1 only weakly stimulates endothelial proliferation. VEGFR1 is expressed not only on vascular endothelial cells but also in monocyte/macrophage-lineage cells. VEGFR-1 is a key receptor in developmental vasculogenesis and angiogenesis (i.e. the formation of vessels during embryogenesis) but does not appear to be critical to pathogenic angiogenesis. Its role appears to vary with stages of development, physiological and pathophysiological conditions and cell type. Thus, the binding of VEGF-A₁₆₅ to VEGFR1 induces directed migration of mononuclear phagocytes across the endothelial cell monolayer as well as their stimulation^{10 12 35}. Macrophages that have migrated via VEGF-A(or PlGF)-VEGFR1 signalling into tumour tissues or inflammatory areas stimulate pathological angiogenesis as well as lymphangiogenesis by secretion of VEGF-A, VEGF-C, and other cytokines¹³⁻¹⁵. In addition, VEGFR1 modulates endothelial cell division at the earliest stages of vascular development just before the formation of the first primitive blood vessels. Mutant mice embryos lacking VEGFR1 (VEGFR1^{-/-}) die at embryonic E8.5-9.0 due to an overgrowth and dysfunction of blood vessels, suggesting a negative role of VEGFR1 in angiogenesis early in embryogenesis¹⁶. Surprisingly, VEGFR1 tyrosine kinase domain-deficient mice (flt-1 TK^{-/-} mice) are basically healthy. Thus, the negative role of VEGFR1 is dependent on the extracellular region, most likely by trapping the endogenous VEGF-A to decrease its local concentration¹⁷. flt-1 TK^{-/-} mice under physiological conditions have no phenotype except for a minor reduction of osteoclasts in bone marrow, but under pathological conditions such as rheumatoid arthritis (RA) and cancer, the levels of malignancy of these diseases are significantly reduced. In the RA model, inflammatory cell infiltration was strongly decreased, and inflammation at the joints was much less extensive in flt-1 TK^{-/-} mice than in wild-type mice. In a tumour transplantation model, tumour growth at local sites decreased accompanied by less infiltration of macrophage and less angiogenesis. Pre-

metastatic induction of MMP9 in distant tissues was about one-third, and the degree of metastasis was significantly reduced in *flt-1* TK^{-/-} mice compared with wild-type mice ³⁶. Treatment of tumour-bearing wild-type mice with anti-VEGFR1 neutralising antibody resulted in a similar reduction in metastasis ³⁷. VEGFR2, also referred to as kinase domain region (KDR) in humans and fetal liver kinase (Flk-1) in mice, exhibits strong tyrosine kinase activity and plays a major role in signalling for angiogenesis. Despite this, its ability to bind with VEGF-A is about one order of magnitude weaker than that of VEGFR1. VEGFR2-signalling is essential for the development of vascular systems in the embryo ³⁸: *flk-1*^{-/-} mice die at E8.5 due to a lack of blood vessels. Activation of the Ras-pathway, which is involved in the signalling of representative tyrosine kinase receptors such as EGFR, is minor in VEGFR2 signalling. Furthermore, a single autophosphorylation site, 1175-tyrosine, is critical for the binding of PLCγ and activation of the PLCγ-PKC pathway ³⁹. The importance of 1175-tyrosine in vivo was proved by the finding that a phenylalanine mutation at this tyrosine in mice (*flk-1* 1173F/1173F mice) induces embryonic lethality at E8.5-9.0 due to a lack of development of blood vessels ⁴⁰. VEGFR2 generates a variety of angiogenic signals not only for endothelial proliferation but for cell migration/morphogenesis including tubular formation. The 951-tyrosine was shown to regulate cell migration by binding with an adaptor, TSA⁴¹. Nrp1, a co-receptor for VEGF-A₁₆₅, binds the basic stretch of VEGF-A₁₆₅, increases the affinity of VEGF-A₁₆₅ for VEGFR2, and enhances signalling ⁴¹.

VEGFR3 is mainly expressed in lymph-endothelial cells, and regulates lymphangiogenesis with its ligands, VEGF-C and -D ^{41 42}. An inactivation mutation at the VEGFR3 tyrosine kinase domain in humans results in familiar lymph-oedema syndrome (Milroy disease) due to insufficient development of lymph vessel systems. The signalling pathway from VEGFR3 is not fully understood yet. PLCγ-PKC pathway was activated after VEGF-C stimulation ⁴¹. On the other hand, activation of the Ras-pathway was also reported. It is of interest to see whether both pathways are equally activated or one of them plays a major role in lymphangiogenesis.

Thus, VEGFR1 binds VEGF-A, and plays a central role in the regulation of angiogenesis, whereas VEGFR3 tightly binds VEGF-C and -D, and stimulates

lymphangiogenesis. The receptor VEGFR 2 binds VEGF, VEGF-C and VEGF-D and promotes the proliferation and motility of endothelial cells. Whilst having highest affinity for VEGF-A ($K_d=15-100\text{pM}$), VEGFR-1 shows 10 fold lower kinase activity than VEGFR-2, and has been proposed to act as a negative regulator of VEGF-induced endothelial cell proliferation ⁴³.

VEGF receptor	Ligand	Function
VEGFR-1	VEGF, VEGF-B, PlGF	Essential for vascular development: VEGF signalling, release of growth factors
VEGFR-2	VEGF, VEGF-C, VEGF-D, VEGF-E	Essential for vascular development: proliferation, migration and angiogenesis
VEGFR-3	VEGF-C and VEGF-D	Proliferation, migration, role in vascular development, signals for lymphangiogenesis

Table 1.2. Roles of VEGF Receptors and their respective Ligands

VEGF is distributed widely in different tissues. Several studies have demonstrated its over-expression in a variety of tumours, including those of breast, ovary, bladder, vulva, uterus and cervix ^{38 44-46}.

Anti VEGF therapy, has been used in clinical trials to inhibit angiogenesis and its use is showing promising results in cancer treatment therapies. Preclinical models of solid tumours have shown anti-VEGF therapy, to cause a slowing of tumour progression, resolution of malignant effusions, and synergy with cytotoxic agents ³⁹.

Bevacizumab, a recombinant humanized monoclonal antibody to VEGF has been shown to induce apoptosis of breast cancer cells and has been used in a combination with 5 Fu for the first line treatment of metastatic colorectal cancer ⁴⁷.

The GOG 170-D is a proof of the principle that bevacizumab is active in Platinum and taxane resistant ovarian cancer ⁴⁸. Other ongoing trials including GOG-218 and ICON-7 are studying the addition the addition of Bevacizumab

to standard frontline chemotherapy. Other agents that target VEGF pathway include VEGF Trap, a soluble decoy receptor ⁴². This causes fusion of the IgG1 Fc ligand binding domains of VEGFR-1 and VEGFR-2. It has been shown to suppress tumour angiogenesis and growth in multiple preclinical models ^{49 50}. It is currently undergoing phase I evaluation.

1.2.2.3 VEGF-independent regulation of angiogenesis

Pro angiogenic Pathways

(a) Platelet Derived Endothelial Growth Factor/Thymidine Phosphorylase

Originally isolated from platelets in 1987 platelet derived endothelial growth factor (PDEGF) is a 47 kilodalton (KDa) protein that promotes cell growth and chemotaxis in endothelial cells in vitro and angiogenesis in vivo ⁵¹. It is a non-glycosylated intracellular protein with no heparin binding activity, found in various tissues such as peripheral lymphocytes, placenta, lung and endometrium ⁵²⁻⁵⁴, as well as in certain cancers, including breast, bladder, colorectal, cervical, endometrial and ovarian tumours^{52 55-57}. The enzyme thymidine phosphorylase (TP) which catalyses the reversible breakdown of thymidine to thymine and 2 deoxyribose phosphate has been identified as being homologous to PDEGF ^{58 59}. The by-product 2-deoxy-D-ribose has been shown to have angiogenic activity ⁶⁰. The role of PDEGF/TP is primarily metabolic, controlling the thymidine levels in the cell. Accumulation of thymidine is toxic to the cells and causes errors in DNA replication, integrity and repair. However high levels of TP have been identified in macrophages ⁶¹, suggesting that it provides other functions such as angiogenic activity. PDGF is an important regulator of angiogenesis via stimulation of the proliferation and survival of smooth muscle cells and pericytes. However in certain culture conditions, PDGF also stimulates endothelial cell growth. It is therefore of interest that whether under pathological conditions or after massive anti-VEGF therapy, PDGF becomes a major player to stimulate the proliferation of vascular endothelial cells or not ³³.

(b) Angiopoietin

The angiopoietins are protein growth factors that promote angiogenesis. There are now four identified angiopoietins: Ang1, Ang2, Ang3, and Ang4. Ang1 and Ang2 are required for the formation of mature blood vessels, as demonstrated by mouse knock out studies ⁶². Angiopoietin 1 (Ang 1) and Ang 2 are growth factors that are ligands for the “Tie” family of receptors. These are tyrosine kinases that are selectively expressed within the vascular endothelium, as are the VEGF receptors ^{63 64}. Although both Ang1 and Ang2 bind Tie2, Ang 1 functions as an agonist whereas Ang2 behaves as an antagonist at this receptor ⁶⁵. Ang2 can cause regression of newly formed vessels by stimulating endothelial cell apoptosis, unless VEGF is present in which case the two collaborate to promote angiogenesis ⁶⁶.

(c) The Ephrin Family

The ephrin (Eph) family represents the largest subgroup of receptor tyrosine kinases, with most vertebrate genomes having 14 members ⁶⁷. Eph receptors can be divided into two classes based on sequence similarity and ligand binding affinity: an A-subclass containing 9 members (EphA1-8, 10) and the B-subclass containing 5 members ^{68 69}. The Ephb2-4 signaling system is relatively specific to the vascular system. It is thought to regulate the differentiation of arteries and veins, being less effective for the proliferation of endothelial cells. Knockout studies have suggested roles for Ephrin B4 and its EphB4 receptors during vascular development, mouse embryo's lacking ephrin B2 and ephrin B4 suffer fatal defects in early angiogenic remodeling that are similar to those seen in mice lacking Ang1 or the Tie2.

(d) Tissue Selective Angiogenic Stimulators

Recently the identification of an angiogenic mitogen selective for one endothelial cell type, the endocrine gland endothelium, has been reported and has been designated endocrine gland derived VEGF ⁷⁰. The expression of this factor is mainly restricted to steroidogenic glands such as the ovary, testis, adrenal cortex and placenta ⁷¹. Although this protein shows no structural homology with the VEGF family, it displays several striking

biological similarities to VEGF; it induces endothelial proliferation and migration and has the ability to induce fenestration in capillary endothelial cells derived from endocrine glands⁷⁰ and it is also regulated by hypoxia.

Angiogenesis Inhibitors

Angiogenesis inhibitors may either be endogenous or exogenous drug or dietary. The endogenous angiogenesis inhibitors are a large, structurally diverse family of protein molecules. Some of these are internal fragments of various proteins that lack any anti-angiogenic activity^{72 73} for example angiostatin is one or more fragments of plasminogen⁷⁴ and endostatin is a fragment of type XVIII collagen⁷⁵. Many of the precursor proteins are components of the extracellular matrix/basement membrane or members of the clotting/fibrinolytic pathways (plasminogen and anti-thrombin III)⁷⁶.

Inhibitors	Mechanism
soluble VEGFR-1 and NRP-1	decoy receptors for VEGF-B and PlGF
Angiopoietin 2	antagonist of angiopoietin 1
TSP-1 and TSP-2	inhibit cell migration, cell proliferation, cell adhesion and survival of endothelial cells
angiostatin and related molecules	inhibit cell proliferation and induce apoptosis of endothelial cells
endostatin	inhibit cell migration, cell proliferation and survival of endothelial cells
vasostatin, calreticulin	inhibit cell proliferation of endothelial cells
platelet factor-4	inhibits binding of bFGF and VEGF
TIMP and CDAI	inhibit cell migration of endothelial cells
Meth-1 and Meth-2	
IFN- α , - β and - γ , CXCL10, IL-4, -12 and -18	inhibit cell migration of endothelial cells, down-regulate bFGF
prothrombin (kringle domain-2), antithrombin III fragment	inhibit cell proliferation of endothelial cells
prolactin	inhibit bFGF and VEGF
VEGI	affects cell proliferation of endothelial cells
SPARC	inhibit binding and activity of VEGF
osteopontin	inhibit integrin signaling
maspin	inhibits proteases
canstatin	
proliferin-related protein	
restin	

Table 1.3. Role of various angiogenic inhibitors

Endogenous angiogenesis inhibitors act as tumour suppressor proteins, analogous to the tumour suppressor p53. Tumour growth in transgenic mice overexpressing endostatin in endothelial cells (a 1.6 fold increase in circulating levels) is 3 fold slower than the tumour growth in wild-type mice ⁷⁷. These mice mimic humans with Down's syndrome who have 1.6 -2.0 fold elevated levels of endostatin as a result of a third copy of collagen XVIII on the trisomic chromosome 21. Individuals with Down's syndrome are the most protected against cancer of all humans ⁷⁸. In these individuals the incidence of all malignant tumours is < 0.1 their expected amount, except for testicular cancer and megakaryocyte leukaemia ⁷⁹. The high circulatory levels of endostatin also protect against two other angiogenesis dependent diseases: retinal neovascularization in diabetes and atherosclerosis ⁸⁰. Individuals with Down's syndrome have the same incidence of diabetes as the general population but have a low incidence of diabetic retinopathy, even with long standing diabetes.

1.2.3 Drug Development in Angiogenesis

1.2.3.1 Angiogenesis Inhibitors

The first angiogenesis inhibitors were reported in the 1980's from the Folkman group ^{81 82}. Angiogenesis inhibitors were not known before the 1980's and there was much speculation amongst the scientific community that such agents would be found. However the drive to isolate and purify them was driven by preliminary data, that led to the 1971 hypothesis that tumour growth is dependent on angiogenesis ³. After the mid 1980's the Folkman group and other groups began to discover additional angiogenic inhibitors ^{74 83-85}. Anti-human VEGF-A neutralising antibody was previously shown to significantly decrease the growth of transplantable human tumours in nude mice, suggesting the blocking of VEGF-A to be useful for the suppression of solid tumour in humans ⁸⁶.

At present there are approximately forty drugs, in which anti-angiogenic activity is central to therapeutic effect, and they have been approved by the FDA in the United States for the treatment of cancer and age related macular degeneration.

Drug	Company	Target	Clinical development
<i>Monoclonal antibodies targeting VEGF-A</i>			
Bevacizumab (Avastin®)	Genentech	VEGF-A	Phase I, II, III
VEGF-Trap	Regeneron	VEGF-A	Phase I
<i>Antibodies targeting VEGFR-2</i>			
IMC-1C11	Imclone	VEGFR-2	Phase I
<i>Receptor tyrosine kinase inhibitors</i>			
SU5416	Sugen/Pharmacia	VEGFR-2	Phase I, II, III
SU6668	Sugen/Pharmacia	VEGFR-2, bFGFR, PDGFR	Phase I, II
SU11248	Sugen/Pharmacia	VEGFR-2, PDGFR,	
c-Kit, Flt-3		Phase I	
PTK787/ZK22854	Schering/Novartis	VEGFR-1, VEGFR-2	Phase I
ZD6474	Astra Zeneca	VEGFR-2, EGFR	Phase I, II
CP-547,632	Pfizer	VEGFR-2, EGFR, PDGFR	Phase I
<i>Inhibitors of endothelial cell proliferation</i>			
ABT-510	Abbott	Endothelial CD-36	Phase I, II
Angiostatin	Entremed	Various	Phase I
Endostatin	Entremed	Various	Phase I
TNP-470	TAP	Methionine aminopeptidase, cyclin dependent kinase 2	Phase I
Thalidomide	Grunenthal	Reduction of TNF- α production	Phase I, II, III
<i>Inhibitors of integrin activity</i>			
Vitaxin	MedImmune	Integrin α V β 3	Phase I, II
Medi-522	MedImmune	Integrin α V β 3	Phase I
Cilengitide	Merck KgaA	Integrin α V β 3	Phase I
<i>Vascular targeting agents</i>			
Combretastatin A4	Oxigene	Endothelial tubulin	Phase I
AVE8062A	Aventis	Endothelial tubulin	Phase I
ZD6126	Astra Zeneca	Endothelial tubulin	Phase I
DMXAA		Induction of TNF-	Phase I

Table 1.4. Angiogenesis inhibitors in clinical trials

The list below includes cancers that are being studied in active Phase 3 treatment clinical trials using angiogenesis inhibitors. The clinical trials are in the National Cancer Institute's (NCI) clinical trials database at <http://www.cancer.gov/clinicaltrials/search>.

- Breast cancer
- Oesophageal cancer
- Gastrointestinal Stromal Tumors (GIST)
- Kidney (renal cell) cancer
- Leukaemia
- Liver (adult primary) cancer
- Lymphoma
- Melanoma
- Multiple myeloma
- Non-small cell lung cancer (NSCLC)
- Ovarian epithelial cancer
- Pancreatic cancer
- Prostate cancer
- Stomach (gastric) cancer

VEGFR is a typical tyrosine kinase; various small chemicals that block kinase activity have been tested in the clinical field and so far two such inhibitors, Sorafenib (Raf and VEGFR inhibitor) and Sunitinib (VEGFR and other tyrosine kinase inhibitors), have been approved for the treatment of renal cell carcinoma. These inhibitors significantly improve the survival of renal cell cancer patients. Furthermore, in late 2007 the FDA approved Sorafenib for the treatment of hepatocellular carcinoma patients based on a clinical study. Anti-angiogenic therapy has recently been reported to significantly increase survival in lung cancer, breast cancer in addition to colorectal cancer (Roy Herbst Lecture ASCO May 2005). Another example is Thalidomide which suppresses the production of endothelial cell precursors and down regulates VEGF; it was approved in Australia in 2003 for the treatment of advanced multiple myeloma and is now used as first line therapy for carcinoma of the breast, ovary, bladder, vulva, uterus and cervix^{38 45 87 88}.

Anti-VEGF therapy has been used in clinical trials to inhibit angiogenesis and its use is showing promising results in cancer treatment therapies. Preclinical

models of solid tumours have shown anti-VEGF therapy to cause a slowing of tumour progression, resolution of malignant effusions, and synergy with cytotoxic agents³⁹. Bevacizumab (Avastin), a humanised anti human VEGF-A neutralizing antibody was the first drug developed solely as an angiogenesis inhibitor, and the first to demonstrate prolongation of survival in patients with advanced cancer. It significantly improved the disease free survival rate as well as the overall survival rate in late stage colorectal cancer patients in a Phase 3 clinical trial and has been FDA approved in 2004 for its use for colorectal cancer⁸⁹. Bevacizumab has also approved for the treatment of non-squamous non-small cell lung cancers, the major type of which is pulmonary adenocarcinoma⁹⁰. The main reason that it has not been applied to squamous-type lung cancer is because of the risk of severe thrombosis and haemorrhage⁹¹. The Gynecologic Oncology Group (GOG) 170-D study is a proof of the principle that bevacizumab is active in platinum and taxane resistant ovarian cancer⁹². Other ongoing trials including GOG-218 and ICON-7 are studying the addition of bevacizumab to standard frontline chemotherapy. Other agents that target the VEGF pathway include VEGF Trap, a soluble decoy receptor that causes fusion of the IgG1-Fc ligand binding domains of VEGFR-1 and VEGFR-2⁴². It has been shown to suppress tumour angiogenesis and growth in multiple preclinical models^{49 50}. It is currently undergoing phase I evaluation.

The increasing availability of approved angiogenesis inhibitors, their relatively low side effect profile and their low incidence of drug resistance, will enable a new approach to cancer based therapy. The evolution and discovery of angiogenesis biomarkers are under development of include

- 1) Quantification of urinary metallinoprotineases⁹³
- 2) Analysis of the platelet angiogenic proteome⁹⁴
- 3) Measurement of blood levels of circulating endothelial cell precursors (CEPS)⁹⁵
- 4) Measurement of mature endothelial cells (CECS)⁹⁵

These methods can detect human tumours in mice at sizes of millimeters or less. In the future, it may be possible to detect recurrent cancer by these and

other biomarkers and to use them to guide anti-angiogenic therapy prior to the onset of recurrent disease or before imaging can detect and or the visualisation of macroscopic tumour is possible.

1.2.4 Angiogenesis and Cancer

Cancer is defined in Wikipedia as ‘a class of diseases in which a group of cells display *uncontrolled growth* (division beyond the normal limits), *invasion* (intrusion on and destruction of adjacent tissues), and sometimes *metastasis* (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumours, which are self-limited, and do not invade or metastasize.’

In a seminal paper based on his own work as well as a number of other studies, Judah Folkman in 1971 proposed that angiogenesis was an important process in tumour growth and metastases ³, and that the inhibition of angiogenesis might be exploited as a means for treating cancer (Folkman 1972 anti-angiogenesis: new concept for therapy). The central point of the hypotheses was that most tumours go through a prolonged state of avascular dormant growth attaining a maximum size of 1-2mm in diameter, the maximum size that tumour cells can obtain the necessary nutrient supplies and oxygen that they require for growth by passive diffusion. These cells, in response to stimulating factors, switch on angiogenesis by recruiting surrounding blood vessels to sprout new vessels which grow toward and infiltrate the tumour mass thus setting the potential for growth and metastases ⁹⁶⁻⁹⁹. Folkman hypothesized that by blocking angiogenesis, established solid tumours could regress to a size of 1-2mm where survival is possible without a blood supply. Therefore the development of tumours is associated with two phases of tumour growth: the pre-vascular phase and the vascular or the angiogenic phase ^{8 97 100}. The pre-vascular phase is seen with various

intraepithelial neoplasia and can persist for many years and is not capable of metastases ⁹⁶. The angiogenic phase occurs when a tumour possesses the innate ability to potentiate its own growth. Oncogenesis of this type whereby the extent of neovascularisation is directly correlated with metastases has been demonstrated in several cancers, for example breast cancer ⁹⁶ and in cutaneous melanoma ⁶. Thus angiogenesis is a crucial factor in the progression of solid tumours and metastases ^{3 101}.

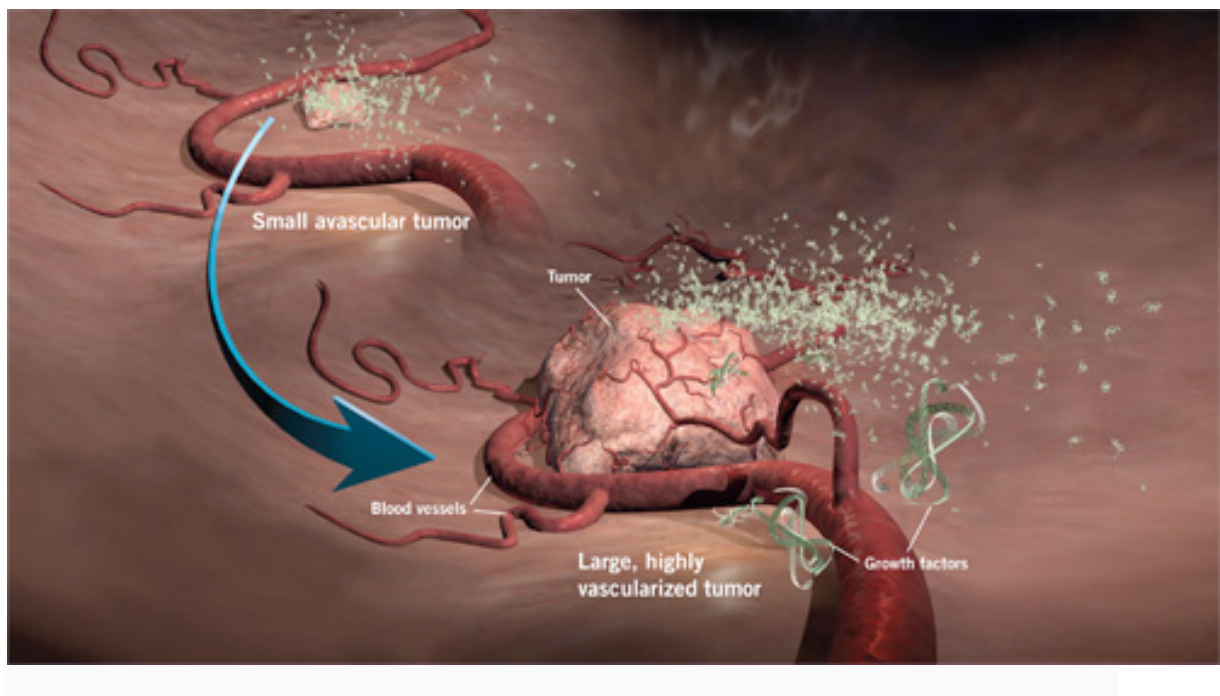


Figure 1.5. Angiogenesis is a hallmark of tumour development: To grow beyond 1 to 2 mm in diameter, a tumour needs an independent blood supply, which is acquired by expressing growth factors that recruit new vasculature from existing blood vessels. This process continues even as the tumour matures. Reproduced from <http://www.biooncology.com/research-education/vegf/multimedia/index.html#resourcePlayer/SlideDecks>

A series of elegant experiments where tumours were transplanted into an avascular environment proved that angiogenesis was a control point in tumour growth: the tumour cells remained dormant indefinitely. However the acquisition of a blood supply resulted in a rapid logarithmic growth ¹⁰².

The normal regulation of angiogenesis is governed by a fine balance between factors that induce the formation of blood vessels and those that halt or inhibit the process. The list of known angiogenic stimulators and inhibitors grows yearly. There are now over 35 activators and 18 suppressants of angiogenesis. While numerous pro-angiogenic factors have been characterised, the VEGF ligand identified by Dvorak et al in 1979 has been identified as the predominant regulator of tumour angiogenesis¹⁰³.

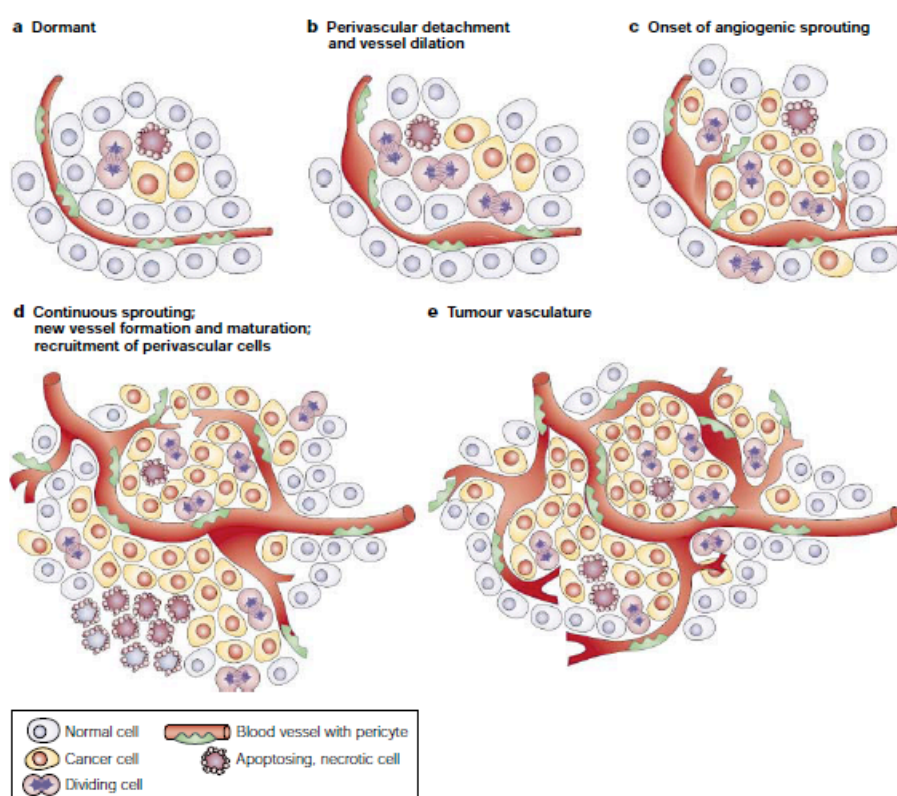


Figure 1.6. The classical angiogenic switch. The angiogenic switch is a discrete step in tumour development that can occur at different stages in the tumour-progression pathway, depending on the nature of the tumour and its microenvironment. Most tumours start growing as avascular nodules (dormant) (a) until they reach a steady-state level of proliferating and apoptosing cells. The initiation of angiogenesis, or the 'angiogenic switch', has to occur to ensure exponential tumour growth. The switch begins with perivascular detachment and vessel dilation (b), followed by angiogenic sprouting (c), new vessel formation and maturation, and the recruitment of perivascular cells (d). Blood-vessel formation will continue as long as the tumour grows, and the blood vessels specifically feed hypoxic and necrotic areas of the tumour to provide it with essential nutrients and oxygen (e)¹⁰⁴.

VEGF is expressed in a wide variety of human malignancies. The VEGF ligand may affect tumour vasculature in 3 essential ways. Early in tumour

development, VEGF may help new vasculature **establish**. Specifically, VEGF has been shown to stimulate tumour growth at both primary and metastatic sites through the recruitment of bone-marrow-derived progenitor cells that form the building blocks of a new vascular network. As this network develops, VEGF may continue to help new vasculature **grow**, providing the blood supply needed to drive further tumour growth and metastasis. Throughout tumour development, VEGF may also help existing vasculature **survive**, allowing tumours to sustain their metabolic requirements over their entire life cycle ¹⁰⁵.

	Helps tumour vessels ESTABLISH	Helps tumour vessels GROW	Helps tumour vessels SURVIVE
Proposed mechanism	Recruitment of progenitor cells to primary and metastatic sites	Stimulation of endothelial cell proliferation, migration, and invasion	Inhibition of endothelial cell apoptosis
Effect on tumour growth	Helps tumour cells seed and form pre-metastatic niches	Provides the blood supply needed for tumours to grow beyond 1 to 2 mm	Maintains a vascular network that fuels continued tumour growth and survival

Table 1.5. Proposed effects of the VEGF ligand

The VEGF ligand is the only angiogenic factor known to be present throughout the entire tumour life cycle. As the tumour develops, it may begin to activate secondary angiogenic pathways, such as basic fibroblast growth factor (bFGF), transforming growth factor beta (TGFβ), placental growth factor (PIGF), and platelet-derived endothelial cell growth factor (PD-ECGF). As these secondary pathways emerge, the VEGF ligand continues to be overexpressed and remains one of the critical mediators of angiogenesis ¹⁰⁶⁻

¹⁰⁹

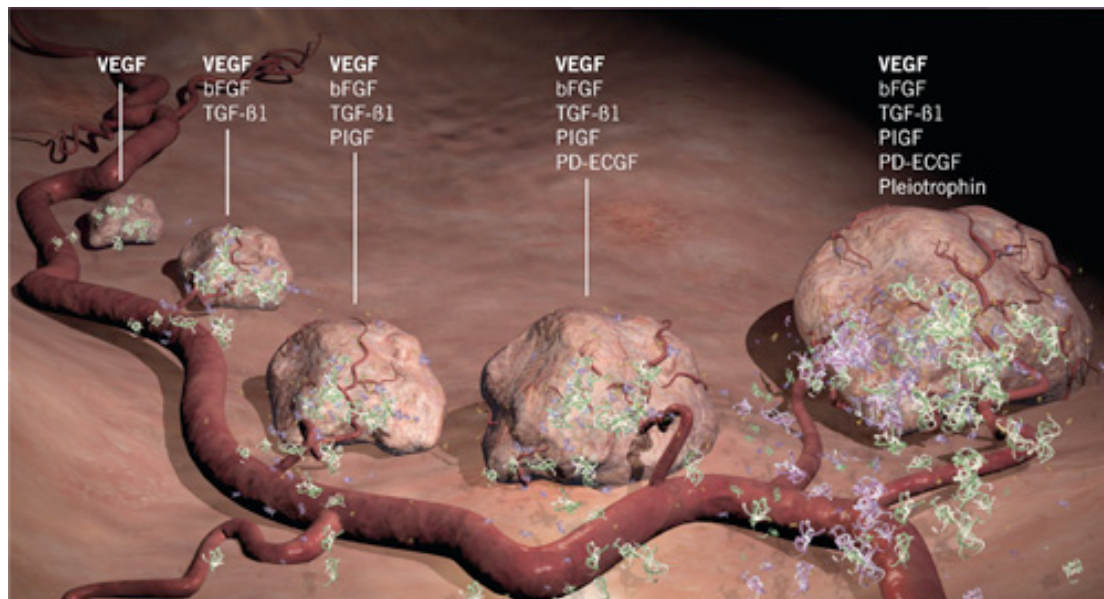


Figure 1.7 VEGF is expressed throughout the tumour life cycle. As the tumour matures, secondary angiogenic factors may be increasingly produced¹¹⁰. Reproduced from <http://www.bioncology.com/research-education/vegf/multimedia/index.html#resourcePlayer/SlideDecks>

The observation that tumours are highly dependent on VEGF early in development and are continuously dependent on VEGF throughout their life cycle is reflected by preclinical research with VEGF inhibitors. In these experiments, VEGF inhibition has demonstrated significant antitumour effects when administered throughout tumour development^{111 112}.

Inhibition of VEGF causes regression of immature vessels but does not disturb normal mature blood vessels. There are physiological differences between mature blood vessels and the immature blood vessels seen in tumour¹¹³. There is extensive endothelial membrane remodeling, leading to tubule and loop vessel formation. The maturation of new vasculature is accomplished by the recruitment of pericytes, which stabilise the new vessels. During normal maturation, growth factors are not needed for endothelium survival. Maturation of the vessels is usually not complete in neoplastic growth; there is constant vascular remodeling leading to microvessel invasion into the tumour stroma, which feeds tumour growth. Endothelial cells of immature blood vessels need growth signals for survival to avoid programmed cell death (apoptosis).

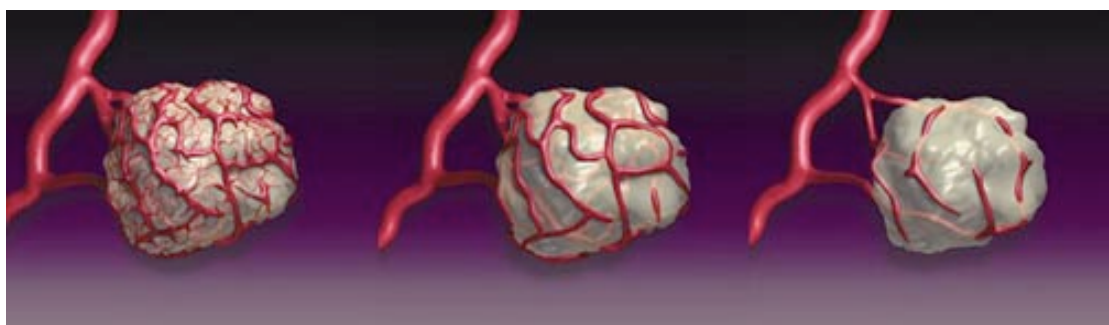


Figure 1.8 By inhibiting the VEGF protein, the blood supply to a tumour may be gradually reduced. Reproduced from <http://www.avastin.com/hcp/overview/resources/moa/index.html>

Physiologically VEGF gene expression is regulated by mutations in the tumour suppressor gene p53 and transient tissue hypoxia, such as that occurring in poorly vascularised areas of tumours ¹¹⁴⁻¹¹⁶.

NORMAL VASCULATURE	TUMOUR VASCULATURE
Organised	Disorganised
Evenly distributed and non permeable	Serpentine and leaky
Properly maintained	Immature
Supporting cells present (pericytes)	Supporting cells deficient
Appropriate expression of membrane proteins	Inappropriate expression of membrane proteins eg integrins

Table 1.6. Histological differences between normal and tumour vasculature

Over-expression of VEGF has been observed across a range of tumour types including colon, lung, breast, renal, ovarian, prostate and other cancers and has been widely associated with tumour development and/ or poor prognosis ¹¹⁷⁻¹¹⁹. Because it drives tumour growth through multiple stages of development, direct and indirect continuous inhibition of the VEGF ligand is a rational anti-tumour strategy.

There are two primary strategies for inhibiting the VEGF pathway; these include inhibiting either the VEGF ligand or the VEGF receptor¹¹⁸. Anti-VEGF strategies that directly target the VEGF ligand include ligand-binding antibodies and soluble receptors. These agents work extracellularly to provide specific inhibition of the VEGF pathway without disrupting other non-VEGF-related targets. Therefore, they may inhibit angiogenesis without affecting other secondary—or “off-target”—pathways^{107 120 121}. Anti-VEGF strategies that target the VEGF receptor include tyrosine kinase inhibitors (TKIs) and receptor antibodies. Agents that target the VEGF receptor intracellularly, such as TKIs, have a wider range of inhibitory effects and may disrupt other secondary pathways that are also mediated through receptor kinases^{107 120 122}.

Finally, although the amount of VEGF that is produced and released may change in response to certain stimuli within the tumour environment, VEGF is thought to be a genetically stable protein that may be relatively unsusceptible to mutation^{118 123}. This genetic stability may make continued targeting of the VEGF ligand a rational antitumour strategy. It is envisaged that a better understanding of these aspects of tumour biology will provide insight into the factors involved in tumour progression and potentially lead us to new therapeutic strategies.

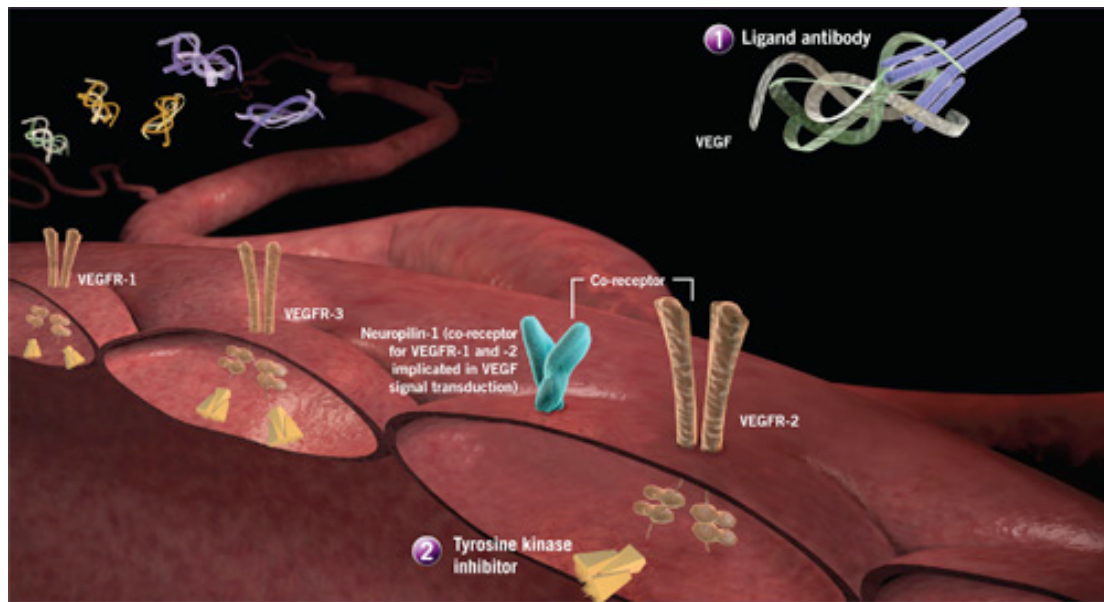


Figure 1.9 Strategies for inhibiting the VEGF pathway¹¹⁸. Reproduced from <http://www.bioncology.com/research-education/vegf/multimedia/index.html#resourcePlayer/SlideDecks>

1. Some anti-VEGF strategies specifically target the ligand, allowing for controlled inhibition of the VEGF pathway.
2. Other strategies target the receptor. Some of these approaches (e.g., TKIs) have a wider range of inhibitory effects and may also disrupt other non-VEGF-mediated pathways.

1.3 OVARIAN CANCER

Ovarian cancer is the fourth most common cancer amongst women in the United Kingdom and the leading cause of gynaecological cancer deaths. In addition, ovarian cancer is the fifth most common cause of deaths in women with over approximately 4400 deaths from the disease every year (Cancer Research UK 2008 Cancer Statistics Registrations 2008).

The natural history of ovarian cancer development, including the nature of the precursor cell type is poorly understood. Approximately 90% of malignant ovarian tumours are epithelial in origin, the remainder are germ cell tumours. Various theories have been suggested alluding to the development of ovarian cancer, including:

1. Incessant ovulation theory ¹²⁴ suggests that the risk of ovarian cancer increases as a result of the recurrent minor trauma to the ovarian surface epithelium that occurs during ovulation. The suggestion is that the greater the number of times the ovarian surface epithelium undergoes repair and regeneration, the greater the chance will be that aberrations leading to a malignant transformation will occur.
2. The gonadotrophin theory¹²⁵ suggests that high levels of gonadotrophins, especially in the early menopausal years, are associated with an increased risk of ovarian cancer as a result of oestrogen or oestrogen precursors stimulating the ovarian surface epithelial lining of ovarian inclusion cysts. Inclusion cysts are benign vacuoles that can form and imbed in the ovarian stroma following repair to the ovarian surface epithelium. Histologically, the epithelial lining of these cyst appear normal.
3. Retrograde transportation hypothesis ¹²⁶ suggests that certain carcinogenic factors, e.g. talc, gain access from the uterus through the fallopian tubes to the ovaries thereby increasing the risk of ovarian cancer.

All of the above theories have been supported by data from epidemiological studies; but no single theory seems has been substantiated thus far. Our understanding of the molecular and genetic events underlying the basis of ovarian cancer development, which may in turn provide support or refute these theories are also poorly understood¹²⁷ .

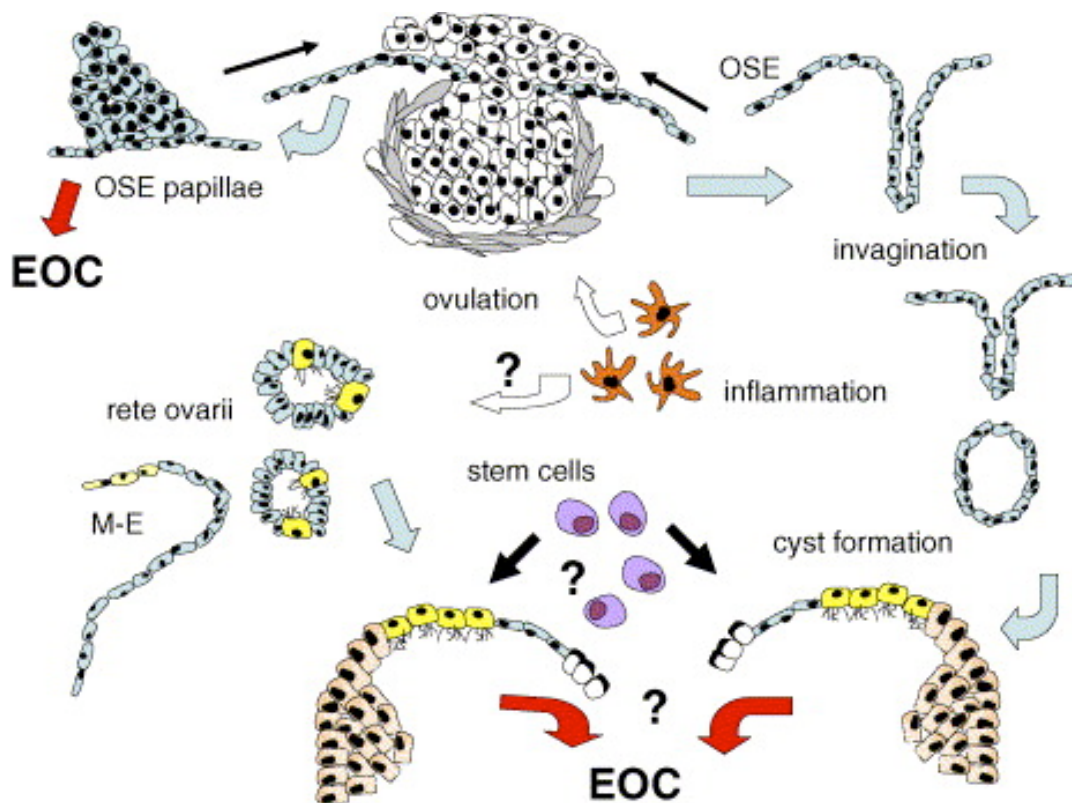


Figure 1.10. Pathways to EOC are influenced by events at ovulation, including hormonal stimulation and inflammation. Post-ovulatory re-epithelialisation involves OSE (blue cells) cell division and possibly migration, to cover the ovulatory lesion (black arrows). Hyperplasia and transformation of the OSE to adenocarcinoma can occur directly (red arrow top left). The pre-ovulatory luteinising hormone surge induces increased expression of cytokines and invasion of macrophages and monocytes (orange cells), leading to differentiation of follicle cells into luteal cells. Ovulation stimulates formation of invaginations and the formation of inclusion cysts. Cyst cells may differentiate to take on müllerian characteristics under the influence of hormonal or cytokine stimulation and become ciliated (yellow cells) or secretory (tan cells), as shown on the right hand side of the figure. Rete ovarii tubules at the hilus of the ovary, close to the mesothelium to OSE transition (M-E), also contain ciliated and secretory cells and can dilate to form cysts, at least in rodents. It is still not known whether cells in both cyst types can transform to become cancerous. Furthermore, the role of any proposed ovarian stem or progenitor cells (purple cells) in epithelial ovarian carcinogenesis remains to be elucidated.¹²⁸

1.3.1 Causes and pathogenesis

Although the subtypes of epithelial ovarian cancer have unique molecular alterations and transcriptional signatures, their morphological features resemble the specialised epithelia of the reproductive tract that derive from the Müllerian ducts. Current research postulates that they might all arise from one surface epithelium precursor cell with specific path of differentiation regulated by embryonic pathways involving *HOX* genes¹²⁹⁻¹³¹.

HOX genes are not usually expressed in ovarian surface epithelium. However, expression of *HOXA9*, *HOXA10*, and *HOXA11* in cells derived from ovarian surface epithelium in tumourigenic mice induces these cells to differentiate along distinct Müllerian lineages, giving rise to tumours with morphological features that are characteristic of serous, endometrioid, and mucinous ovarian tumours, respectively. *HOXA7* controls extent of differentiation and grade of ovarian tumours. Since sex steroids regulate *HOX* expression throughout the menstrual cycle, protracted exposure of ovarian surface epithelium cells to these hormones in adult women might contribute to inappropriate *HOX* activation, perhaps in the context of epithelial inclusion cysts and excessive autocrine or paracrine stimulation leading to proliferation and genomic instability.

Genomic mutations have a vital role in the development of many forms of cancer. High-prevalence somatic (non-germline) mutations (>5%) have been reported in only a small number of genes in epithelial ovarian cancer. Like the *HOX* gene changes, these mutations occur in a histological subtype-specific and grade-specific manner, and identify genes whose functional perturbation probably has a role in ovarian carcinogenesis. These genes include *TP53*, *CTNNB1*, and *PTEN* (all inactivated), and *KRAS*, *PIK3CA*, and *AKT1* (all activated)^{129 132 133}.

Hereditary (germline) *BRCA1* and *BRCA2* mutation-related epithelial ovarian cancers tend to occur at an earlier age than do sporadic tumours and are

more often high-grade serous tumours with P53 dysfunction ¹³⁴. Like many solid tumours, epithelial ovarian cancers frequently have a high amount of chromosomal instability (gene copy number amplifications and deletions), and both total and regional instability are associated with tumour grade and patient outcome ¹³⁵. Although such aberrant areas of DNA frequently carry many genes, only a small number of genes are thought to be key drivers of the process; these key drivers are the most crucial markers and potential treatment targets. Since inhibition of protein function is generally believed to be more achievable than is restoration of function, most investigators focus on areas of chromosomal gain (amplicons) for identification of novel potential targets for therapy. Candidate drivers at areas of copy number gain that have been identified in ovarian tumours include *RAB25* at 1q22, *EVI1* (ecotropic viral integration site-1), *PRKCi* (protein kinase C iota), *SKIL*, *BCL6*, the initiation factor *EIF5A2*, and *PIK3CA* at 3q26·2, *MYC* and *PVT1* at 8q24·2, *RSF1* (remodeling and spacing factor 1) and *PAK1* at 11q13, *HER2* (also known as *ERBB2*) at 17q12, *AKT2* at 19q13·2, and *ZNF217*, *AURKA* (Aurora Kinase), *PTK6*, and *EEF1A2* at 20q13·2 ^{86 98 136}.

Some of these genes can be targeted with novel agents that are in preclinical or early clinical assessment. Rearrangements, epigenetic changes, and imprinting also affect cellular function and identify potentially important markers and therapy targets.

In one model of ovarian carcinogenesis, epithelial ovarian cancers are divided into two categories that are designated type I and type II tumours, corresponding to two main pathways of tumourigenesis ^{96 127 130}. Type I tumours arise in a stepwise manner from borderline tumours and include low-grade serous carcinomas, mucinous, endometrioid, and clear-cell carcinomas. Type II tumours arise de novo and include high-grade serous carcinoma, malignant mixed mesodermal tumours, and undifferentiated carcinomas. Type II tumours are characterised by frequent *TP53* mutations, genomic instability, and *BRCA* mutations in some cases. This model of carcinogenesis reconciles the relation of borderline tumours to invasive carcinoma and provides a

morphological and molecular framework for studies aimed at elucidating the pathogenesis of epithelial ovarian cancer ¹²⁶.

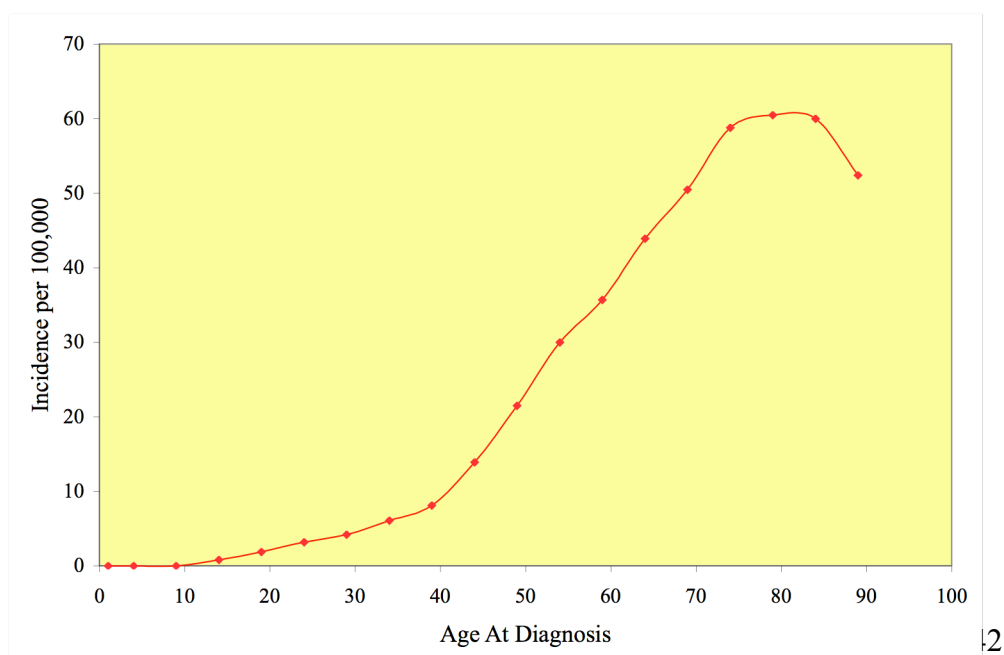
Recent evidence, have led to suggest that both type 1 and type 2 tumours develop from extraovarian tissue, from the fallopian tube, and the fallopian tube peritoneal junction, rather than the ovarian surface epithelium, that was previously believed ¹³⁷
¹³⁸.

1.3.2 Epidemiological and Genetic Risk Factors

1.3.2.1 Age

The single biggest risk factor for ovarian cancer is age. There is a progressive increase in ovarian cancer incidence with age. For epithelial ovarian tumours, the risk of disease in women under the age of 30 is low even in families with evidence of a hereditary basis of ovarian cancer. From 30 to 50 years of age, ovarian cancer incidence increases in a linear fashion. It then continues to increase at a lower rate, reaching a maximum incidence of 60.5 per 100,000 in the 75 to 79 year age group (data from the US Surveillance, Epidemiology and End Result).

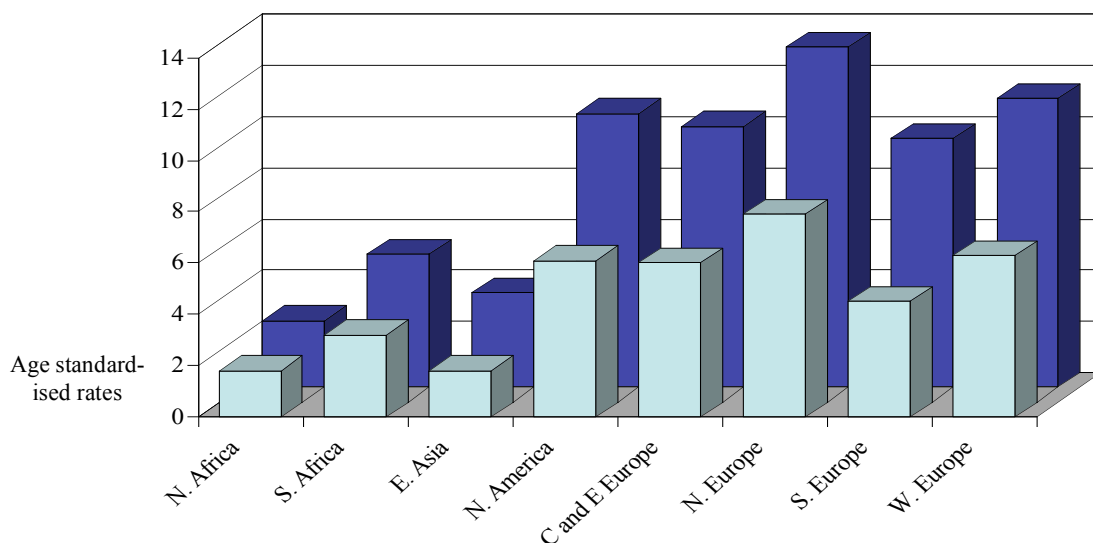
Figure 1.12 Age-associated incidence of epithelial ovarian cancer (data from the Surveillance Epidemiology and End Results Program of the National Cancer Institute)



1.3.2.2 Geography

Ovarian cancer incidence varies widely across different geographic regions and ethnic groups (see Figure 3). The highest incidence is in Northern Europe (14. cases per 100,000 women in Sweden) and the United States (13.3 cases per 100,000 women); the lowest incidence is in Japan (2.7 cases per 100,000 women). In Pakistan the risk of Ovarian cancer is similar in incidence to the West, with an ASR of 10.1 per 100,000. As with other cancers, there are obvious increases in risk in populations that migrate from a country with low risk to a country of higher risk indicating a possible role for dietary and environmental factors.

Figure 1.12: Geographical variation in incidence and mortality rates for epithelial ovarian cancer (data from the GLOBOCAN 2002 database project hosted by the Descriptive Epidemiology Group at the International Agency for Research on Cancer)



1.3.2.3 Family history

The majority of ovarian cancer cases are sporadic, and the lifetime risk of developing ovarian cancer is between 1 -2 %. However, 5-10% of cases of all ovarian cancer cases have a hereditary basis (i.e. they are familial). Despite this, the single greatest ovarian cancer risk factor is a family history of the disease; the relative risk of ovarian cancer in a woman with an affected first degree relative is 3.1 (95% CI:2.6-3.7). The level of ovarian cancer risk is correlated with the number of affected first and second-degree relatives and the age at diagnosis- the highest risk is associated with women below the age of 50 that have a first degree affected relative under 50.

There are at least two groups of individuals with a hereditary predisposition to ovarian cancer for which pedigree analyses suggest autosomal dominant transmission with variable penetrance. Families with the BReast CAncer 1 and 2 (BRCA1 and BRCA2) mutations are responsible for approximately 90 % of families with strong history of ovarian cancer (more than 3 cases in first degree relatives) or multiple cases of ovarian and breast cancer. The lifetime risk of developing ovarian cancer for BRCA1 carriers is 16-44% and for BRCA2 carriers 27% ¹³⁹⁻¹⁴².

The BRCA1 gene is located on chromosome 17q21; it consists of 22 coding exons distributed over 100kb of genomic DNA. It has 5592bp of coding sequence and encodes a protein of 1863 amino acids. Approximately 80% of the mutations are frame shift or nonsense mutations causing truncation of the protein ¹⁴³. In 1994 Wooster et al localised the second breast ovarian cancer gene at chromosome 13q12-13 ¹⁴⁴. The BRCA2 gene consists of 26 coding exons distributed over approximately 70kb of genomic DNA. It has 10254bp of coding sequence and encodes a protein of 3418 amino acids. There are approximately 100 separate mutations described thus far, and as is for BRCA1, these are scattered throughout the coding sequence and, apart from several distinct founder mutations ¹⁴⁵.

BRCA1 and BRCA2 both function as tumour suppressor genes, and are involved in specific pathways of DNA repair and are likely to be involved in specific carcinogenic pathways in conjunction with other genes ¹⁴⁶. Therefore the inactivation of BRCA1 or BRCA2 may lead to an increased frequency of

DNA breaks, leading to alteration of other genes involved in growth regulation and transformation and thus predisposing to an increased susceptibility to cancer ¹⁴⁷.

Ovarian cancer is also part of the phenotype of hereditary non-polyposis colorectal cancer (HNPCC) syndrome (alternatively known as the Lynch syndrome), which is commonly associated with inherited susceptibility to colorectal, gastric and endometrial cancers. The lifetime risk of ovarian cancer for a carrier in a HNPCC family is about 10%¹⁴⁸. HNPCC families are caused by mutations in one of several genes that function in DNA mismatch repair pathway.

High-risk genes that cause ovarian cancer are rare and are responsible for only 30% of the excess familial ovarian cancer risk. It is likely that the remaining familial risks are the result of more common but less penetrant genetic variation (moderate risk genes); but these genes await identification

1.3.2.4 Reproductive And Hormonal Factors

Early menarche and late menopause, have been implicated as risk factors in the development of ovarian cancer, however there have been several epidemiological studies that have looked at age at menarche as a risk factor for ovarian cancer. In general, these have found no association ¹⁴⁹⁻¹⁵³.

Although no association has been found between age at menopause ovarian cancer risk for most studies ¹⁵¹⁻¹⁵⁴, a small number of studies have suggested that late menopause may increase risk with estimates ranging from 1.5 to 2.9 fold increased risks in oldest menopause groups compared with younger referents ^{150 153 155}.

1.3.2.5 Parity

Low parity is a risk factor for ovarian cancer. The association between the number of pregnancies and a decreased risk of ovarian cancer is well established. Epidemiological studies have continually shown that parity is protective against ovarian cancer. Whittemore et al ¹⁵¹ reviewed 12 case-

control studies and showed that parity was significantly protective effect against ovarian cancer; there was an approximately 40% reduction in risk with first birth and a further reduction of 10% with each subsequent birth.

There may also be an association with the age at first birth, although this is less clear. Some hospital-based studies suggest that an older rather than younger age at first birth is associated with greater risk, but case-control studies with population based controls indicate the reverse is true ^{150 156 157}.

Whilst the impact of term pregnancies on the ovarian cancer risk is clear, the effect of miscarriages, terminations and ectopics is not. A case-control study from Denmark found no relationship between ovarian cancer and pregnancies that fail to go to term ¹⁵⁸. However, other studies, suggest that incomplete pregnancies confer some risk reduction, albeit a weaker protective effect than for full term pregnancies ^{152 153 157}.

1.3.2.6 Lactation

Most studies that have separated the effects of breast-feeding from pregnancy have demonstrated a small protective effect from lactation. Risk estimates range from between 0.6 and 0.9 in parous women who have breastfed their children compared with those who have never breastfed ^{151 157 159 160}.

1.3.2.7 Oral contraceptive pill

Based on a large body of epidemiological studies, it is now accepted that the oral contraceptive pill (OC) protects against ovarian cancer. The cause of this protective effect has been put down to the cessation of ovulation and/or the decrease in gonadotrophin levels in mid-cycle. In case-control and prospective studies, 'ever' users of OCs have been shown to have a lower risk compared to never-users ^{150-152 155 161}.

The protective effect increases with duration of OC use; there is a 10-12% decrease in risk associated with a one year OC use ¹⁶² and an approximate 50% decrease after 5 years of use ¹⁶³. The risk reduction associated with OC

use continues for a long time after cessation of the OC; several studies showed a 40-70% risk reduction even 10 years after cessation of OC use¹⁵⁰
151 155 164

One recent study even suggested a risk reduction after 25 years of OC use¹⁵⁰. OCs confer a protective effect regardless of other known risk factors such as parity or age^{161 164}. However, there does appear to be an additive effect for parity and OC use combined; Franceschi et al found that women who have two children and have taken the oral contraceptive pill for ≥ 5 years had a 70% risk reduction for ovarian cancer¹⁶⁵.

The risk reduction for OC use may also be associated with different histological sub-type of ovarian cancer. In a case-control study that examined the effect of OC use on the risk of mucinous and non-mucinous ovarian cancer, by Risch et al found that the risk of mucinous ovarian cancer was not reduced in women on the combined oral contraceptive pill¹⁶⁶.

There are a wide variety of oral contraceptives with differing content of oestrogens and progestins. The initial OCs of the 1960s were high dose monophasic formulations. Hormonal doses were then reduced in the 1970s and in the 1980s biphasic and triphasic formulations were introduced. The majority of studies showing the protective role of OCs were based on women using the early monophasic formulations. The protective effect appears to be present in newer formulations as well; use of one of two types of low dose OC formulations ($\leq 35\mu\text{g}$ of ethinyl oestradiol) compared to never-users was associated with a reduced relative risk of ovarian cancer of 0.7 and 0.4 respectively and there was a risk reduction with multiphasic OCs as well. In another study, in which both high and low dose OCs reduced the risk of ovarian cancer, the high-dose regimen appeared slightly more effective¹⁶⁷.

A few studies that have evaluated the effect of progesterone only contraceptives on ovarian cancer suggest a protective effect. In a study of 5000 women receiving medroxyprogesterone injections with a 4-13 years follow-up, there was an insignificant decrease in ovarian cancer risk (RR 0.8, 95%CI 0.1-4.6)¹⁶⁸.

The association between oral contraceptive use and ovarian cancer risk in women who are BRCA carriers has also been studied. In a population-based study, no association was observed between oral contraceptive use and risk

reduction in high-risk women ¹⁶⁹. However, in a family based study, a 60% risk reduction was observed in women with BRCA mutations who had been on the pill for 6 or more years ¹⁷⁰. More recently, in a study of 451 BRCA1/2 mutation carriers, the odds-ratio for ovarian cancer associated with the use of oral contraceptives for 6 or more years was 0.62 (95% CI 0.35-1.09) after adjusting for parity ¹⁷¹.

1.3.2.8 Infertility

In 1992, a collaborative analysis of 12 US case control studies reported that the risk of ovarian cancer in nulliparous women who received fertility treatment was increased 27 fold. However, this finding should be treated with caution for two reasons. Firstly the confidence intervals for this study were wide (95% CI 2.3-315.6) ¹⁷¹. Secondly, the individual studies that make-up the collaborative differ vastly in the depth with which the relevant information was collected; only 3 of the 12 studies contained results regarding infertility therapy. Since this report, a further two case-control studies have failed to find an association between fertility drug use and ovarian cancer ^{171 172}.

A number of cohort studies of women undergoing fertility treatment have also failed to show an increased ovarian cancer risk associated with infertility ¹⁷³

In the largest of these studies, the excess risk of ovarian cancer was observed in women with unexplained fertility that had not had any fertility drugs ¹⁷³.

There are several difficulties in study design that make this a difficult question to address, and this may be responsible for some of the disparity observed between studies. For example, it is unclear whether the risk of ovarian cancer increases as women come to an age where ovarian cancer is more common, which coincides with the timing of infertility treatment. In addition, for case-control studies, there are problems associated with defining the 'infertility type', the different types of fertility drugs used and in the selection of an appropriate control group.

1.3.2.9 Hormone replacement therapy

Issues relating to the use of hormone replacement therapy (HRT) and its safety continue to challenge clinicians.

HRT initially contained oestradiol or conjugated oestrogens only. It then became apparent in the 1970s that the use of oestrogen therapy (ET) was associated with an increased risk in endometrial cancer. As a result, progestins were added to the ET in women with an intact uterus. ET, however, continues to be used in women who have undergone a hysterectomy.

Studies on the effect of ET/HRT on the risk of ovarian cancer are contradictory. In a recent cohort study that followed 44,241 menopausal women for approximately 20 years, a relative risk of 1.6 (95% CI 1.2-2.0) was observed among ever-users compared with never users of ET ¹⁷⁴. The largest risk observed in this study was for women who used ET for 20 years or more: the relative risk was 3.2 (95% CI 1.7-5.7). In another study, there was an increased risk of ovarian cancer associated with ET of 10 or more years ¹⁷⁵.

Until recently many of the studies that examined the effect of combined HRT on ovarian cancer risk have been too small to draw firm conclusions. One such study suggested that HRT did not increase the risk of ovarian cancer if progestin was used for more than 15 days per month ¹⁷⁶. The largest trial so far on the effect of HRT on ovarian cancer risk is the Women's Health Initiative (WHI) ¹⁷⁷. In this double blind randomised control trial approximately 17,000 women were randomised to either combined HRT or placebo. After an average 5.6 years of follow-up, there was a non-statistically significant increase in ovarian cancer risk in users of HRT compared to the placebo group (hazard ratio 1.58, 95% CI 0.77-3.24).

1.3.2.10 Talc powder

There is some evidence to suggest that agents that irritate and inflame the ovarian epithelium promote ovarian carcinogenesis. This theory arose from observations that asbestos was associated with mesotheliomas in animals ¹⁷⁸ and that particle passage from the vagina to the ovary was possible ¹⁷⁹. Talc

powder use in the genital area has been postulated to increase the risk of ovarian cancer by ascending the genital tract. This theory has been supported in a case control study ^{179 180} that gave an OR of 1.6 (95% CI of 1.18-2.15) and that talc use was associated with serous and undifferentiated tumours.

1.3.2.11 Pelvic surgery

The association between pelvic surgery such as tubal ligation and hysterectomy and ovarian cancer has been reported in a number of epidemiological studies. Although the Oxford Family Planning Association study showed no association between sterilisation and ovarian cancer, (OR 1.5 [95% CI 0.7-3.1]) ¹⁸¹, the majority of studies support a protective effect with observed risk reductions from 10-80% ¹⁸²⁻¹⁸⁵.

A similar protective effect was observed in women who underwent hysterectomy, although the magnitude of protection appears to be lower than that of tubal ligation ¹⁸²⁻¹⁸⁵.

Both these operations provide closure of the ovaries to the external genital tract and it has been suggested that these operations reduce the risk of ovarian cancer by preventing carcinogens from ascending the genital tract. It is however interesting that the protective effect has been reported only up to 20 years after surgery.

1.3.2.12 Endometriosis

Pathology and epidemiological studies have consistently shown an association between endometriosis and ovarian cancer, particularly of the endometrioid ^{186 187} and clear cell subtypes of ovarian cancer. Histopathology studies analysing large series of ovarian tumours have identified ovarian endometriotic lesions in 5-10% of cases. These were most commonly found in tumours of the endometrioid (up to 60%) and clear cell (up to 15%) sub-types, which is disproportionate to the expected frequencies of these sub-types of ovarian cancer (10-20% and 3-10% respectively). In another study, endometriosis was found in 40% of women with stage I

endometrioid or clear cell carcinoma, one third of which were carcinomas arising out of the endometriotic lesions. Two theories have been proposed for the transformation of endometriosis to ovarian cancer. Firstly, aberrant inflammation may serve to promote the growth and invasion of ectopic endometrium. Secondly, it has been postulated that the same balance of steroid hormones that has been shown to increase the severity of endometriosis may also enhance the occurrence of ovarian cancer.

1.3.2.13 Polycystic ovarian syndrome (PCOS)

Clinical features of PCOS commonly include obesity, infertility, menstrual abnormalities and hirsutism. In addition PCOS is also characterised by a raised luteinising hormone (LH) to follicle stimulating hormone (FSH), increased androgen production and abnormal oestrogen secretion. There is a well-established relationship between PCOS and endometrial cancer risk but the risks associated with ovarian cancer are less clear. In a case-control study¹⁸⁸, the risk of ovarian cancer was increased in women with PCOS (OR 2.5; 95% CI 1.1-5.9) and the risk was greater in women who had not used the OC (OR 10.5; 95% CI 2.5-44.2). Other studies, however, found no association between PCOS and ovarian cancer¹⁸⁹.

1.3.2.14 Pelvic inflammatory disease

Pelvic inflammatory disease (PID) can arise as a complication of sexually transmitted diseases or after childbirth, terminations and gynaecological procedures. Whilst some studies have found a positive association between PID and the risk of ovarian cancer¹⁹⁰ others have not^{152 183 191}. In a Canadian study, there was an increased risk of ovarian cancer with one episode of PID compared to those with none (OR 1.5, 95% CI 1.0-2.1). Risks were also greater if PID had occurred at an earlier age, if the women were nulliparous, infertile or had repeated episodes of PID¹⁹⁰. Despite the association between human papilloma virus (HPV) and cervical cancer, no association has been found with ovarian cancer^{192 193}.

1.3.2.15 Diet

Diet may affect ovarian cancer risk but there appears to be no consensus about which dietary factors may be causative or protective. Several studies have suggested a link between one or more of lactose, animal fat, meat, egg and cholesterol intake with an increased risk of ovarian cancer^{194 195}.

A high consumption of vegetables and olive oil on the other hand may decrease risk¹⁹⁵⁻¹⁹⁷. A systematic review of 11 population based case-control studies and 5 cohort studies¹⁹⁸ showed a positive association between body size and ovarian cancer risk, which is of course associated with dietary and calorific intake. These findings have been confirmed in more recent studies¹⁹⁹⁻²⁰³.

1.3.3 Clinical Features of Ovarian Cancer

The clinical manifestations of this malignancy are non-specific and show great variability. Pain and abdominal distention provide the most obvious complaints either because of the primary ovarian mass, ascites or epigastric omental plaque. The gastrointestinal manifestations of ovarian malignancy include dyspepsia, vomiting and alteration of bowel habit. Irregular periods, shortness of breath and urinary problems are also symptoms of the disease. Owing to the silent nature of the initial stages of the disease, there is little correlation between symptoms and the stage of the disease. In most cases the disease presents at an advanced stage (FIGO stage III and IV).

Manifestations of extra abdominal disease can include nodal metastases (especially inguinal or supraclavicular), pleural effusions and uncommon deposits in breast, lung, bone, or skin, including the umbilicus (Sister Joseph's nodule). Pleural effusions alone does not allocate a patient to stage IV disease unless malignancy is confirmed by exfoliative cytology. Rarely

respiratory symptoms may dominate the clinical picture and an ovarian primary source may be occult or even undetectable.

Bilateral involvement of the ovaries is common, but it is unclear whether this is due to metastatic spread or multicentric origin. Possible routes of spread to the opposite ovary include transperitoneal seeding, cell migration through the tubes and endometrial cavity and lymphatic spread via anastomoses between ovarian and uterine lymphatic vessels. The uterus is frequently involved with tumour. Extension to the endometrium may be via the lymphatics or by luminal migration of tumour cells through the tube. In advanced stages of ovarian cancer the peritoneal wall, diaphragm and omental structures are seeded with micro and macrometastases of tumour cells (transcoelomic spread). Lymphatic dissemination to the pelvic (48-80%) and para-aortic lymph nodes (58-78%) ²⁰⁴ are also common especially in advanced disease. Ascites is associated with ovarian cancer of stages III and IV. Ascitic fluid arises as a plasma exudate, its formation resulting from an imbalance between the influx and efflux of fluid from the peritoneal compartment (Nage 1993).

Ovarian cancer patients show increased plasma levels of CA 125, a low molecular weight glycoprotein found in tissues derived from fetal embryonic coelomic epithelium ²⁰⁵. Serum levels up to 35U/ml are considered normal. Normal ovarian epithelium does not express CA 125. Elevated levels are found in 85% of women presenting with ovarian cancer, but this is reduced to 50% when only those with stage I disease are considered ²⁰⁶. Unfortunately it is also elevated in a number of other conditions including fibroids, endometriosis, inflammatory conditions involving the pleura and peritoneum, and cirrhosis, which can lead to false positive results ²⁰⁷.

1.3.3.1 Histopathology of Ovarian Tumours

Epithelial ovarian cancers are classified by histopathological grade (grade 1–3) and appearance (serous being the most common, mucinous, endometrioid, and, less commonly, clear cell, transitional, squamous, mixed, and

undifferentiated subtypes). Additionally, fallopian tube and primary peritoneal cancers occur that morphologically and clinically resemble epithelial ovarian cancers, possibly because the same embryonic precursor is shared by the ovarian surface epithelium and the peritoneal and fallopian tube epithelia. There is some evidence that suggests that some ovarian cancers might originate from the distal tubes (fimbria) ²⁰⁸.

In a series of more than 8000 cases, mucinous and endometrioid carcinomas were associated with a favourable prognosis, serous carcinomas less so, and undifferentiated carcinoma was the most aggressive subtype ²⁰⁹. Data for clear-cell carcinoma were conflicting. Grade has consistently been of prognostic significance.

1.3.3.2 Grading of ovarian cancers and prognostic significance

The grade of the ovarian cancer refers to the cells themselves. The most important features in assessment of grade are mitotic activity, nuclear size and pleomorphism, and differentiation from the original cells ²¹⁰. Grading is not entirely objective, and different pathologists may disagree on the grade of a particular ovarian cancer, as there are different systems for grading ovarian cancers²¹¹. Pathologists usually use a combination of the pattern system and Broder's grading system.

There are three different grades; grade 1 (well differentiated), grade 2 (moderately differentiated), and grade 3 (poorly differentiated) ²¹². Tumours are often heterogeneous, and the grading is normally performed on what appears to be the least differentiated area²¹¹. However in view of the extent of variation it is usually based on the worst features. Overall low-grade (grade 1) tumours grow more slowly, and have a better prognosis than high-grade (grade 3) tumours ²¹⁰. However, determining prognosis is multi-factorial ²¹².

1.3.3.3 Histological subtypes of ovarian cancers

Ovarian tumours may be divided into five broad categories (Underwood, 2000) according to the World Health Organisation :

- Epithelial
- Germ-cell
- Sex-cord stromal
- Metastatic
- Miscellaneous

1.3.3.4 Epithelial Ovarian Cancer

Malignant epithelial tumours of the ovary are adenocarcinomas and account for over 90% of malignant tumours. They are thought to originate from the epithelium on the surface of the ovary, which is coelomic in origin ^{213 214}. More recent evidence suggests that epithelial tumours arise from the fallopian tube and tubo peritoneal junction ¹³⁸ Epithelial ovarian cancer is subdivided into six categories.

a) Serous carcinoma is the most common type of epithelial ovarian cancer, accounting for 40-50% of all cases. Tumours consist of cuboid epithelial cells. Well-differentiated serous adenocarcinomas have a predominately papillary pattern. Often some areas show an irregular acinar pattern. Poorly differentiated serous adenocarcinomas have a predominately solid histological appearance, with sheets of small relatively uniform cells and poorly formed glandular acini. Tumours are often bilateral and multicystic.

b) Endometrioid Carcinomas account for 20% of epithelial ovarian cancers. Typically oval, columnar cells form tubular gland-like structures similar to those seen in endometrial cancer. Papillae are blunter and broader than in serous adenocarcinoma. Stromal invasion is usually apparent.

c) Mucinous Carcinomas account for 10% of all epithelial ovarian cancers. They are characterised by tall columnar mucus secreting cells which tend to have large multiloculated cystic structures filled with thick viscous fluid. Tumour cells can appear to be floating in large cells of mucous.

d) Clear Cell Carcinomas account for 5-10% of epithelial ovarian cancers. Architectural patterns vary considerably. Tumours may be papillary, acinar or solid, with polyhedral cells containing clear cytoplasm and angular nuclei. Tubules and cysts are common.

e) Brenner tumours are the rarest ovarian epithelial cancers, accounting for only 1-2% of all epithelial ovarian tumours. Areas of squamous differentiation are common. Nests of urothelial-like epithelial cells with a dense collagenous stroma are typically seen.

f) Unclassifiable/ Undifferentiated: 17% of epithelial ovarian cancer are undifferentiated and behave in an aggressive fashion.

1.3.4 Management of Ovarian Cancer

Primary debulking surgery for advanced ovarian cancer followed by platinum based chemotherapy has been the standard of care for the last 3 decades. Subsequently, in the 1990s, taxanes were added to platinum based chemotherapy.

The International Federation of Gynaecology and Obstetrics (FIGO) staging system is given below. It is based on the concept that ovarian cancer spreads first to sites within the pelvis and then to the peritoneal cavity, only metastasizing outside the peritoneal cavity in advanced disease.

Table 1.7 Staging Of Ovarian Cancer. International Federation of Gynecology and Obstetrics criteria (2002)

FIGO STAGE	DISEASE EXTENT
I	Tumour limited to ovaries
IA	Tumour limited to one ovary; capsule intact; no tumour on ovarian surface; no malignant cells in ascites or peritoneal washes.
IB	Tumour limited to both ovaries, capsule intact, no tumour seen on ovarian surface; no malignant cells in ascites or peritoneal washings
IC	Tumour limited to one or both ovaries, malignant cells in ascites or peritoneal washings with any of the following; capsule ruptured, tumour
II	Tumour involves one or two ovaries with pelvic extension .
IIA	Extension and/or implants on the uterus and/or tube(s). No malignant cells seen in ascites/peritoneal washings
IIB	Extension to other pelvic tissue; No malignant cells in ascites/ peritoneal washings.
IIC	Pelvic extension (IIa and IIb); malignant cells seen in ascites /peritoneal washes.
III	Tumour limited to one or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis.
IIIA	Microscopic peritoneal metastases beyond the pelvis
IIIB	Macroscopic peritoneal metastasis beyond the pelvis 2cm or less in greatest dimension.
IIIC	Peritoneal metastasis beyond the pelvis more than 2cm in greatest dimension and/or lymph node metastasis
IV	Distant Metastases (excludes peritoneal metastases)

1.3.4.1 Surgical Management

Primary surgery has for many years been the mainstay of the initial management of advanced ovarian cancer. This has been based on the work of Griffiths identifying the completeness of cytoreductive surgery as a prognostic factor for outcome^{215 216 217}. Peritoneal washings are usually taken immediately on entering for cytology. A thorough exploration of the abdomen and pelvis is performed to assess the extent of spread. The surface of the liver, diaphragm, and spleen are examined, as well as the small and large bowel, omentum, para-aortic and pelvic lymph nodes. A total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, para-aortic and pelvic lymph node sampling, peritoneal biopsies, and peritoneal cytology (peritoneal washings) are usually performed if ovarian cancer is confirmed. In advanced disease cytoreduction or debulking surgery (where as much tumour tissue as possible is removed) is performed, which will then offer the best response to subsequent chemotherapy. It is recommended to achieve a residual disease of less than 1cm^{218 219}.

The survival advantage of surgical debulking or cytoreduction for advanced-stage disease has been shown retrospectively in studies as early as 1934^{219 220}. In a meta-analysis of 53 studies including 6885 patients with stage III or IV disease, Bristow and colleagues reported that every 10% increase in cytoreduction was associated with a 5.5% increase in median survival. The parameter that defines an optimum cytoreduction and allows maximum survival advantage remains debatable, although the most widely accepted definition is residual disease less than 1 cm²¹⁹⁻²²¹. Optimum cytoreduction is often established as no visible residual disease because prognosis is directly related to the number of residual implants and to the size of residual disease^{219 222 223}. The role of a systematic dissection of retroperitoneal lymph nodes remains debatable and might be appropriate in patients for whom maximum cytoreduction is possible^{224 225}. Although most experts agree that optimum cytoreduction improves survival, whether aggressive or ultra-radical surgery (e.g., diaphragm or hepatic resection, splenectomy, cholecystectomy, distal pancreatectomy, peritonectomy, modified posterior exenteration) increases survival in patients with extensive tumour is unknown²²⁶.

Retrospective data suggest that aggressive surgical resections can increase rate of optimum debulking and thereby correlate with improved outcomes, albeit at the expense of increased complication rates ^{227 228}; for example, pneumothoraces and symptomatic pleural effusions occur in roughly 20% of women after diaphragmatic resection ²²⁸. A 2004 consensus statement from the Third International Gynecologic Cancer Intergroup Ovarian Cancer Consensus Conference (GCIG-OCCC-2004) unanimously recommended an initial maximum surgical effort at cytoreduction, with the goal of no residual disease ²²⁹. Specialist gynaecological oncologists at high-volume centres are best suited to debulk tumours in patients with advanced disease ^{220 230}. Surgery done by a gynaecological oncologist resulted in a 5-8 month survival advantage for these patients ²³¹. The GCIG-OCCC-2004 unanimously recommended that surgery in cases of advanced disease be done by an appropriately trained surgeon skilled in management of epithelial ovarian cancer ²²⁹.

1.3.5 Prognostic Factors

The surgical management of patients with epithelial ovarian cancer facilitates accurate staging and in turn allows the evaluation of a series of clinicopathological variables that are often used to select postoperative therapy. The established clinicopathological prognostic factors are described below.

1.3.5.1 Tumour Stage

The 5-year survival of patients with epithelial ovarian cancer is directly correlated with the tumour stage at diagnosis (Cancer Research UK):

- Stage 1 – 90% 5 year survival
- Stage 2 – 70% 5 year survival
- Stage 3 – 21% 5 year survival
- Stage 4 – 6% 5 year survival

1.3.5.2 Volume of Residual Disease

The volume of residual disease following cytoreductive surgery directly correlates with survival ^{232 233}, Delgado 1984). Patients who have been optimally cytoreduced have a 22 month improvement in median survival compared to those patients undergoing less than optimal debulking. In studies outlining the importance of residual volume upon survival, the size of the largest residual tumour mass ²³⁴ and not the total number of lesions has been thought to be the primary factor correlating with prognosis. Yet the number of residual masses may be an important prognostic factor as well ^{235 236}; Patients who have only a single residual mass following cytoreductive surgery have a significantly greater chance of achieving a surgically confirmed complete remission compared with those patients with multiple small nodules even though each nodule is less than 2cm in size. In addition to the volume of disease other unknown biological factors influence survival in patients with advanced disease, as patients with bulky disease versus small volume disease at the outset prior to cytoreduction have a worse overall survival. To accommodate this, the FIGO stage III disease is subdivided into three groups based upon the volume of disease. This permits a comparison of survival for patients who have small volume disease prior to cytoreductive surgery (IIIA – IIIB) with that of patients who present with bulky disease (IIIC).

1.3.5.3 Histological Subtype and Grade

In general, the histological type has less prognostic significance than the other clinical factors, such as stage, volume of disease and histological grade. In some series, patients with mucinous adenocarcinoma have an overall better survival in comparison to endometrioid or serous adenocarcinomas. These findings reflect the rarity with which high-grade mucinous adenocarcinoma of the ovary is diagnosed. Endometrioid carcinoma also has been suggested to have a better prognosis than serous adenocarcinoma, as well as presenting with a lower histological grade and clinical stage. Some studies have shown

that ovarian clear cell adenocarcinoma may be more aggressive than the other common epithelial malignancies on a stage for stage basis²³⁴

The histological grade of the tumour is a particularly important prognostic factor in patients with early stage disease. Stage I patients with well or moderately well differentiated tumours have a greater than 90% 5 year survival when treated with surgery alone²³⁷. In contrast, patients with stage I disease with poorly differentiated or clear cell tumours have a significantly worse survival, and postoperative adjuvant chemotherapy is indicated. In advanced stage patients treated with platinum based chemotherapy, most studies have failed to demonstrate a significant correlation between histological grade and survival (Pettersson F Annual Report on the results of treatment in gynaecological cancer vol 20 1988). This may reflect variable degrees of intra-observational and inter-observational variation in grading of ovarian tumours^{238 239}

1.3.5.4 Surgical Prognostic Factors

Surgical factors such as tumour size, bilateralism and ascites without cytologically positive cells are not thought to be of prognostic significance in patients with early stage disease. However tumour spillage, capsular penetration and cytologically malignant ascites (FIGO stage IC) are associated with a worse prognosis.

1.3.5.5 CA 125 Levels

The prognostic significance of preoperative and postoperative CA 125 levels has been established^{240 241}. Serum levels of CA 125 generally reflect the volume of disease. Pre-chemotherapy CA 125 levels have been shown in univariate analysis to be of prognostic significance. In multivariate analysis, they are usually not an independent prognostic factor owing to their association with volume of disease²⁴¹. In addition preoperative high CA 125 levels may confer worse overall survival. Postoperative CA 125 levels appear to have greater prognostic significance. In a multivariate analysis,

postoperative CA125 levels were of independent prognostic significance in patients with or without residual disease²⁴²

Furthermore CA 125 levels are useful for predicting disease response to chemotherapy. Criteria have been proposed to define response to treatment based on serum CA 125 levels²⁴³⁻²⁴⁵. An elevated CA 125 has also been increasingly used as an indicator of progression following completion of chemotherapy. Clinical trial groups have established criteria for the progression of the disease based on elevations of CA 125 levels or the observance of physical or radiographic evidence of disease.

Table 1.8 Prognostic and predictive factors in early-stage and advanced stage epithelial ovarian cancer

Early-stage (stages I and II)

- Stage (IA-IB vs. IC vs. II)
- Rupture of the ovarian capsule (when considering stage II disease)
- Grade
- Histotype
- Age
- Pelvic fluid cytology (positive vs negative)

Advanced-stage (stages III and IV)

- Residual tumour size after surgical debulking (≤ 1 cm vs >1 cm)
- Stage (III vs IV)
- Histological subtype
- Age
- Grade
- Lymph-node involvement

*

1.3.6 Chemotherapy for epithelial ovarian cancer

The majority of patients with ovarian cancer present with advanced disease and although response rates to chemotherapy are high, many patients eventually develop drug resistant tumours and the 5-year mortality from ovarian cancer remains at 60-70%.

There are many chemotherapeutic agents used to treat ovarian cancer, depending on the type, grade, stage of the disease, and the health of the patient. Meta analyses of randomised clinical trials have shown that platinum compounds (cisplatin/carboplatin), anthracyclines (doxorubicin/epirubicin), alkylating agents (cyclophosphamide/ melphalan), spindle poisons (paclitaxel/docetaxel), anti-metabolites such as gemcitabine, and the topoisomerase I inhibitor topotecan are the most active agents in ovarian cancer ²⁴⁶.

1.3.6.1 Spindle Poisons

Spindle poisons include taxanes such as paclitaxel and docetaxel, and promote the assembly of microtubules by binding to β -tubulin, which inhibits depolymerisation ²⁴⁷. This action disturbs mitosis in normal and malignant cells ²⁴⁸. The taxanes were originally derived from the bark of the Pacific Yew tree, *Taxus brevifolia*, and paclitaxel was identified as the active constituent in 1971²⁴⁹ (McGuire et al., 2003). Docetaxel is a semi-synthetic taxoid derived from the needles of *T. baccata* ²⁵⁰ and has been shown to be less neurotoxic than paclitaxel ²⁵¹. The introduction of paclitaxel in the 1990's to treat ovarian cancer increased the survival rates of women with the disease ²⁵².

1.3.6.2 Anthracyclines

Anthracyclines such as doxorubicin and epirubicin, have several cytotoxic actions. Their main mechanism of action appears to be mediated by their effect on topoisomerase II, whose activity is markedly increased in dividing cells ²⁵³. Topoisomerase II binds to the double stranded DNA, cleaves both strands of duplex DNA and passes a second duplex through this transient cleavage, involving hydrolysis of ATP, and enzyme recycling ²⁵⁴. The intermediate form is termed the 'cleavable complex'. As a result of its double-stranded DNA passage mechanism, topoisomerase II is able to remove negative or positive superhelical twists (i.e., under- or overwinds) from the

genetic material and resolve intramolecular DNA knots as well as intermolecular tangles²⁵⁵.

Doxorubicin poisons the cleavable complex, inhibiting religation of the cleaved complex, resulting in double strand breaks of the DNA. Doxorubicin intercalates into the DNA and becomes trapped²⁵⁶. Apoptosis results if the cell is unable to repair these DNA double-strand breaks. However, these drugs have additional modes of action such as production of oxygen free radicals, and formation of covalent adducts which may contribute to its toxicity²⁵⁷.

1.3.6.3 Topoisomerase inhibitors

Topoisomerase I inserts a nick into one strand of the DNA to relax supercoiled DNA that occurs when DNA replicates itself. It also re-ligates the cleaved DNA. Irinotecan and topotecan (9-dimethylaminoethyl-10-hydroxycamptothecin) are potent inhibitors of topoisomerase I²⁵⁸.

In patients with platinum-resistant ovarian cancer, there was a low probability of responding to single agent Topotecan²⁵⁹. Unfortunately Topotecan in combination with gemcitabine in the treatment of platinum refractory ovarian cancer in a phase I/II trial and has shown disappointing results²⁶⁰.

1.3.6.4 Antimetabolites

Antimetabolites such as gemcitabine, interfere with nucleic acid production, either by mimicking nucleotide structure, enabling them to be incorporated into nucleic acids, thus terminating their production, or by preventing synthesis of nucleotides directly.

Gemcitabine (2', 2'-difluorodeoxycytidine dFdC) is a fluorinated deoxycytidine derivative, which can be used in a variety of solid tumours including ovarian cancer (usually in combination with carboplatin), non-small cell lung cancer, head and neck cancer, and genitourinary cancer²⁶¹. Gemcitabine enters the cell through a membrane nucleoside transporter²⁶², and it is activated by phosphorylation to gemcitabine monophosphate, which is subsequently

phosphorylated to the 5'-diphosphate (dFdCDP) and triphosphate (dFdCTP), which represent the active forms of gemcitabine. dFdCTP is incorporated into DNA instead of cytosine, followed by one or more deoxynucleotides, thus blocking DNA synthesis and preventing repair by 3'-5'-exonuclease activity. Gemcitabine triphosphate is also incorporated into RNA, thus terminating RNA synthesis. Only a proportion of gemcitabine is converted into the di- or triphosphate forms. The majority of gemcitabine is rapidly inactivated in the blood, liver and kidneys by deamination into 2', 2'-difluorodeoxyuridine in a reaction catalysed by deoxycytidine deaminase (CDD) (Fruscella et al. 2003).

1.3.6.5 Platinum compounds cisplatin and carboplatin

Cisplatin (cis-dichlorodiammine platinum II) was discovered serendipitously by Rosenberg in the 1950's while investigating inhibition of bacterial growth by electric currents²⁶³. The resulting inhibitory complexes were produced by the reaction of platinum electrodes with growth media. Cisplatin (a neutral complex) was the most active of these, and was tested in clinical trials²⁶⁴.

1.3.6.6 Current First Line Therapy

The main stay of first line therapy for ovarian cancer is platinum based chemotherapy combined with paclitaxel. The value of adjuvant therapy for advanced disease is well established (NHS Executive 1999). Platinum drugs are the most active agents in untreated ovarian cancer and form the mainstay of any regimen for this disease. The survival benefit for platinum therapy is particularly impressive in population based studies although the effect of platinum on overall survival in individual randomised trials is not large²⁶⁵⁻²⁶⁸.

When cisplatin was introduced into first line trials in ovarian cancer there was little difficulty in recognising its impact on progression free survival (PFS). The FDA approved cisplatin in its approval indication for ovarian cancer in 1979. Although its impact became increasingly clear, its initial use was hampered by complex combinations. Later on with the development of anti-emetics and simplification of treatment regimes, cisplatin became the treatment of choice

following surgery for all stages of ovarian cancer. Studies by the Gynecological Oncology Group (GOG) employed it in combination with cyclophosphamide and this became first line treatment until GOG 111 showed the PFS and overall survival (OS) superiority of cisplatin and paclitaxel ²⁶⁹. Eventually in terms of reduced toxicity, GOG 158 showed that carboplatin and paclitaxel was preferred because of less toxicity with at least equal efficacy to cisplatin ²²³.

The current standard treatment is to give cisplatin or carboplatin in combination with paclitaxel ^{269 270}. Women with stage III or IV ovarian cancer have between 6-8 cycles of intravenous platinum-paclitaxel chemotherapy after primary surgery. If the patient was not optimally debulked at primary surgery, interval debulking after 3- 4 courses of chemotherapy may be of use. Failure of Ca 125 levels to return to normal after primary surgery and initial chemotherapy has been associated with the degree of residual disease remaining and a likely adverse disease course. The interval between the end of first line therapy and first relapse, or the treatment free interval between subsequent relapses is an important determining factor for response.

The role of other cytotoxic drugs in current use for ovarian cancer in complementing the effects of the platinum drugs is less clear. After exposure to cisplatin various alkylating agents had disappointing activity ²⁷¹, and Doxorubicin's acute and chronic toxicities led to diminishing use until the advent of liposomal formulations. Upon the introduction of Paclitaxel, followed by topotecan, gemcitabine, and pegylated liposomal doxorubicin (PLD) drugs with reproducible activity and reasonable therapeutic index came into use ²⁷². However the addition of the latter three drugs in five arm GOG 182 did not prove advantageous over the combination of carboplatin and paclitaxel ²⁷³. Radiotherapy has very limited use in ovarian cancer.

1.3.7 Anti-angiogenic and new 'targeted' agents

These drugs are a major focus of current therapeutic research. The anti-tumour activity of bevacizumab in ovarian cancer has led to the study of this agent in the first line and recurrence strategies for ovarian cancer ^{274 275}.

Activity has been shown with anti-angiogenic approaches other than bevacizumab and the efforts to establish their role is ongoing. Sorafenib added to bevacizumab has shown promise in a phase I study, but at a cost of enhanced toxicity ²⁷⁶. Efforts to combine them with first line platinum based regimes or to test a strategy of maintenance following initial surgical and platinum based cytoreduction are undergoing evaluation in studies currently.

1.4 Angiogenesis in Ovarian Cancer

Recurrent VEGF-mediated angiogenesis followed by vascular regression is unique to the ovulatory cycle and is highly regulated ²⁷⁷. New vessel generation is essential for the whole reproductive cycle, and the role of vascular growth factors involved in regulating this process, particularly VEGF, has been demonstrated by highly regulated changes in expression throughout an ovulatory cycle ²⁷⁸.

Early in tumourigenesis, the previously highly maintained balance of pro-angiogenic and anti-angiogenic factors that regulate normal ovarian function is tipped, with the down regulation of anti-angiogenic signalling and the up regulation of pro-angiogenic growth factors ²⁷⁸. Although VEGF is expressed in healthy ovaries, it is overexpressed in ovarian hyperstimulation syndrome and ovarian cancer ^{277 279}. VEGF expression is hypothesized to mediate multiple functional and structural characteristics of the disease and angiogenesis plays an important part in the growth and metastasis of ovarian cancer ²⁸⁰ and several studies have shown that VEGF regulated angiogenesis is an important component of EOC growth.

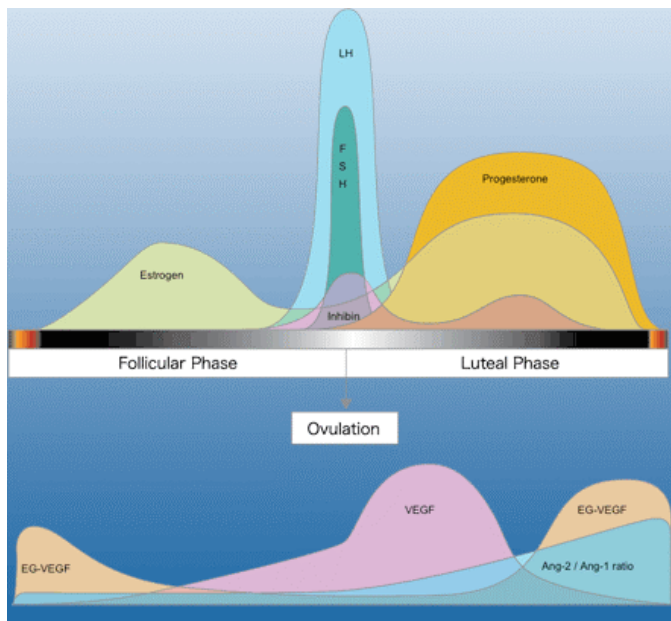


Figure 1.13 Hormonal profiles and angiogenic responses during a human ovulatory cycle. Intrafollicular levels of VEGF, in particular VEGF-A, increase during the initial part of the ovulatory cycle, peaking just before the start of the luteal phase.² Subsequent vascular regression during follicular atresia is promoted by elevated levels of angiogenic inhibitors and reduced VEGF levels toward the end of the luteal phase and the ovulation cycle. LH=luteinising hormone; FSH=follicle-stimulating hormone; EG-VEGF=endocrine-gland derived-vascular endothelial growth factor (Ramakrishnan et al. 2005, Kumaran, Jayson and Clamp 2009).

The degree of angiogenesis of a tumour, as assessed by microvessel density (MVD), has emerged as a powerful candidate for prognosis and as a predictive tool²⁸¹. In a multivariate analysis, MVD was found to be the most accurate prognostic indicator in breast carcinoma for disease free survival; better than size, grade, or oestrogen receptor status²⁸². In other studies, VEGF expression has been demonstrated in a variety of tumours and has been correlated with increased MVD and poor prognosis²⁸³⁻²⁸⁵.

MVD and degree of expression of VEGF and its receptors in ovarian tumours are directly correlated with poor prognosis suggesting that angiogenesis influences disease progression in EOC²⁸⁶⁻²⁸⁸. The role of angiogenesis in ovarian carcinoma was uncertain until Hollingsworth *et al.* conducted a retrospective study on 43 advanced-stage epithelial ovarian cancer patients²⁸⁹. Their results suggested that the degree of neovascularization was associated with overall and disease-free survival and may be a useful prognostic factor. Using a Cox proportional hazards model, Hollingsworth concluded that stage was the best predictor of overall survival. Tumour angiogenesis, however, was found to be the best predictor of disease-free survival. This study did not include women with early-stage disease and contained a small number of study subjects.

An investigation by Abulafia *et al.* evaluated angiogenesis in 42 consecutive patients with primary epithelial ovarian cancer. In 19 patients with advanced disease, the degree of neovascularisation was assessed in both the primary tumour as well as metastatic omental implants ¹³⁵. Overall, the microvessel counts of the primary tumour were not significantly related to patient age, preoperative CA 125 level, tumour stage, tumour grade, or patient survival. In contrast, in women with advanced stage disease, the microvessel counts of the omental metastases were significantly correlated with preoperative CA 125 level and were significantly predictive of survival. This study also contained a small number of subjects, and only 12 patients had early-stage disease.

Ovarian cancer is characterized by the rapid growth of solid intraperitoneal tumour cells creating large areas of hypoxia and large volumes of ascitic fluid ²⁹⁰. The growth of ovarian tumour cells is supplied by ascites, and ascites accumulation is associated with progression ²⁹¹. Experimental models have demonstrated a positive correlation between ascites volume and VEGF expression ²⁹².

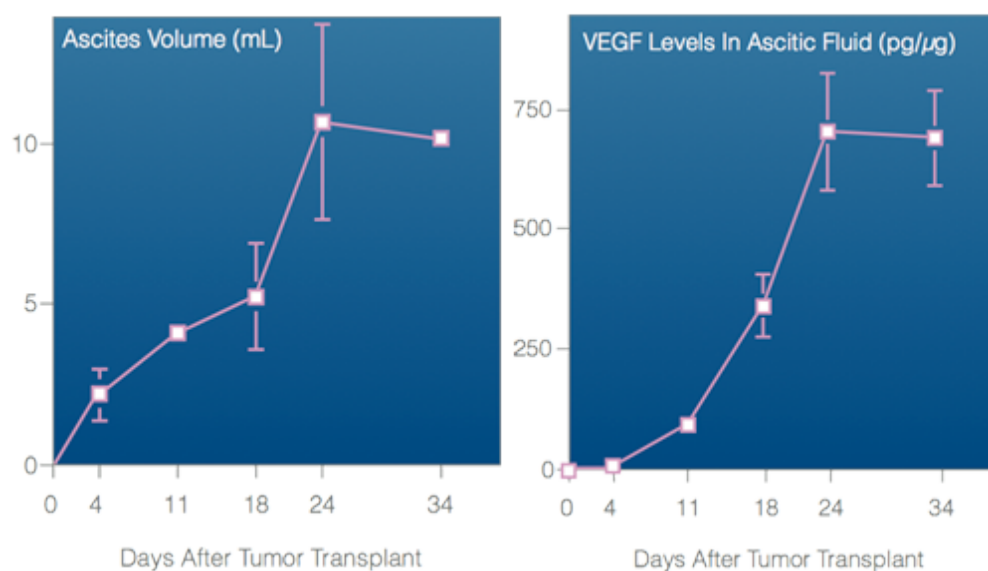


Figure 1.15. Correlation between ascites volume and VEGF expression. In a murine model, a positive correlation was observed between ascites volume and VEGF levels in ascitic fluid. In this same study, a correlation was also observed between ascites volume and tumour burden ²⁹².

Ovarian malignancies are presumed to metastasize through the process of exfoliation of tumour cells from the primary tumour, followed by dissemination of cells throughout the peritoneal cavity, implantation, and subsequent growth. Most patients who succumb to this disease ultimately die from starvation and inanition due to bowel obstruction caused by intraperitoneal metastases. In this regard, the degree of angiogenesis of ovarian cancers may directly influence the clinical course of the disease. Highly angiogenic tumours may: (a) facilitate a rapidly increasing volume of tumour cells, thereby facilitating the dissemination of the cells within the abdominal cavity and accelerating the clinical development of bowel obstruction; (b) provide access to the circulatory system; and (c) promote the development of lymphatic channels. Conversely, a lack of angiogenic capability in ovarian tumours may serve to prevent the rapid development of a large intraperitoneal tumour burden, which is a prerequisite for bowel obstruction, thereby resulting in improved median survival for women with ovarian carcinoma, and angiogenesis has been correlated with poor prognosis in patients with advanced ovarian cancer²⁸⁹.

1.4.1 VEGF expression in ovarian cancer

VEGF is expressed in a wide variety of human malignancies and is considered to be an important mediator of angiogenesis especially in ovarian cancer²⁹³. In ovarian cancer, VEGF appears to be a prognostic factor as well as helping monitor the clinical course of the disease²⁹⁴. Patients with disseminated cancer have higher serum levels of VEGF compared to patients with localised disease and healthy controls²⁹⁵. Mesiano et al have shown higher levels of VEGF in the ascitic fluid of patients with high stage tumours²⁹⁰. Barton et al reported an increased concentration of VEGF in the ascitic fluid and serum²⁹⁶. The formation of ascites in ovarian cancer seems to be directly associated with VEGF expression²⁹⁷. VEGF mRNA has been found in the majority of epithelial ovarian cancers in the malignant cells but not in the normal stroma, although the protein does accumulate in the stromal

matrix²⁹⁸. In xenograft models of human EOC, the expression of VEGF is associated with the formation of ascites and the tumour growth rate is proportional to the MVD, which correlates to VEGF²⁹⁷. As seen with other tumours, human EOC overexpress VEGF, and its expression is directly correlated to MVD²⁹³.

Wong et al showed that VEGF expression was significantly more pronounced in malignant vs. benign tumours, and borderline tumour and normal ovary^{299 300}. There was a significant difference in VEGF expression between the histological subtypes; serous subtypes of benign and malignant tumours expressed higher amounts of VEGF. Serous adenocarcinomas had significantly higher VEGF expression compared with endometrioid carcinomas. It was also shown in this study that the degree of VEGF expression was related to the stage of EOC, late stage EOC, having higher VEGF expression when compared with early stage EOC. This may indicate that VEGF is involved in the process of invasion and angiogenesis in serous tumours.

1.4.2 VEGF Expression and its relationship to prognosis in ovarian cancer

Overall, VEGF expression and high degrees of tumour angiogenesis in ovarian cancer have been associated with poor survival outcomes²⁹¹.

Expression of VEGF in ovarian carcinomas has also been associated with tumour growth and proliferation within the peritoneal space, and intense VEGF staining is more often found in peritoneal metastases than in primary tumours²⁷⁷.

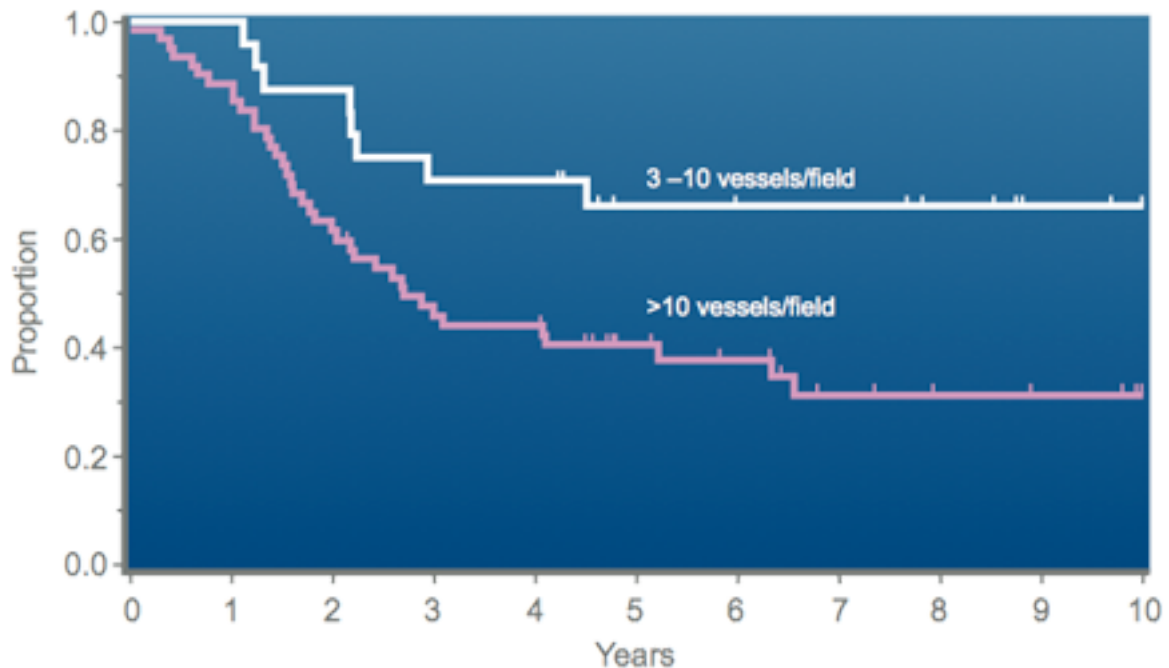


Figure 1.16. Microvessel density (MVD) and overall survival in ovarian cancer. The degree of angiogenesis, as measured by MVD, is associated with prognosis (as measured by the proportion of patients surviving). In this experiment, survival in patients with high MVD (>10 vessels/field) was significantly lower than in patients with low MVD (3 to 10 vessels/field) ²⁹¹.

There have been many studies examining the prognostic value of VEGF in EOC; Paley et al illustrated that VEGF expression was a significant and independent predictor for shorter relapse free survival and overall survival³⁰¹. Wong te Fong et al showed that survival rates of patients with EOC whose tumour was positive for VEGF were significantly worse than those of patients with VEGF negative tumours ^{302 303}. In a recent study by Engels et al, it was shown that the expression of VEGF-A is a prognostic marker for clinical outcome in serous ovarian cancer patients with microscopic complete tumour resection (R0)³⁰⁴. These studies illustrate that VEGF may have an important role to play in the prognosis of EOC, as a negative predictor for patients with early and late stage cancer.

1.4.3 PDEGF/TP in ovarian cancer

It has been shown that angiogenesis is associated with normal ovarian, follicular and corpus luteal development. Studies have shown that PDEGF/TP expression increased from normal, benign, borderline and malignant ovarian tumours and high PDEGF/TP expression is correlated with poor survival⁶¹. importance in the progression of early ovarian carcinomas and may also have some prognostic relevance. Nakanishi et al found that the patients with advanced ovarian cancer showed an increase of PDEGF/TP expression in stromal cells²⁹³. Similarly Hata et al showed that PDEGF/TP was significantly higher in EOC specimens than in normal ovary specimens³⁰⁵.

1.4.4 The Role of MVD assessment in ovarian cancer

Several techniques have been applied to assess the extent of angiogenesis in clinical samples. Simple measurements of microvessel number and or density have been done using immunohistochemical methods^{135 306-308}. Endothelial cells can be identified by antibodies to von Willebrand's factor, factor viii, CD 31 and CD 3. High microvessel counts have been correlated with relapse of disease in all stages of ovarian cancer²⁸⁹.

The extent of angiogenesis has been shown to correlate with progression free survival and overall survival in ovarian cancers. Hollingsworth et al studied advanced stage ovarian cancer and identified CD 34 as the most useful discriminant of microvessel density; MVD counts and stage of disease were associated with overall survival and disease free survival, respectively²⁸⁹.

The expression of VEGF has been used to complement microvessel density in the clinical assessment of angiogenesis. Paley and colleagues first demonstrated that higher expression of VEGF correlated with poor outcome in early stage EOC and LMP ovarian tumours³⁰¹. Although variability in methods exists, neoangiogenesis is a consistent clinical predictor of poor progression

free survival in EOC, in univariate and multivariate analysis. However measures of angiogenesis have not proven consistently as an independent variable for overall survival.

Using anti-vWF, Orre et al showed that average microvessel counts in malignant serous and benign ovarian tumours were similar and significantly less compared with other markers³⁰⁹. This was thought to result from the reduced or even absent expression of vWF factor antigen in the smaller less mature microvessel of many tumours, therefore giving conflicting results as to the importance of MVD in EOC tumourigenesis³¹⁰.

It has been found that in EOC, angiogenesis was equally stimulated regardless of the stage of the disease²⁹³; Nakanishi et al suggested that angiogenesis might be induced differently, depending on the organ involved and the histological type of the tumour. It is postulated that angiogenesis is necessary for cancer cell growth and allows tumours to increase in volume; other mechanisms also play a crucial role in tumour progression in EOC. It was suggested that degree and control of angiogenesis may differ between ovarian tumour types^{289 311}. Following on from this observation, angiogenesis in EOC has also been shown to be intensified with invasive capability and tumour vascularity. This was shown in a study by Abufalia et al and it was felt that the assessment of tumour vascularity by immunohistochemical techniques of MVD quantification, may help the gynaecologist, and pathologists to differentiate between borderline and invasive tumours³¹².

In solid tumours, including EOC there is a significant correlation between the incidence of metastasis and MVD^{289 293 312}. These areas are thought to represent ongoing tumour angiogenesis, in addition to the site of tumour entry into the circulation³¹³. It is however postulated that ovarian tumours spread via peritoneal dissemination rather than through the vasculature⁹⁶, tumour angiogenesis is unlikely to play a role in this type of spread. Weidner et al showed that MVD as assessed by immunohistochemical staining for endothelial cells was an independent prognostic factor in breast cancer MVD has since been reported as a possible prognostic indicator/factor in numerous human solid tumours including breast³¹⁴, prostate¹²⁹, non-small cell lung cancer³¹⁵, gastric³¹⁶ and colorectal cancers³¹⁷. The reports and studies

assessing the role and prognostic value of MVD in advanced EOC are conflicting as studies have shown that angiogenesis may not be as pertinent, as that seen in other types of carcinomas in which angiogenesis is an independent prognostic factor. In a recent study by Rubatt et al ³¹⁸ which looked at the expression of cd105 (Endoglin), this is expressed almost exclusively on proliferating endothelial cells, as opposed to CD31 and CD34 which are pan endothelial markers and stain nearly all blood vessels including the established mature vessels. As CD105, recognises proliferating vessels it may be a better prognostic marker³¹⁹. The study showed that high MVD as assessed using CD 105, a marker of proliferating endothelial cells and neoangiogenesis but not CD 31, a pan endothelial marker, appeared to be an independent prognostic factor for worse progression-free survival in women with advanced EOC, after adjusting for prognostic clinical covariates.

Others have reported that MVD a marker of tumour angiogenesis has prognostic significance in ovarian cancer, with most studies reporting that a high degree of tumour angiogenesis conferred a worse survival^{289 291 320-322}. Only one study found that high MVD was a prognostic factor for improved survival in advanced ovarian cancer³²³ and one study did not find any evidence of an association between MVD and survival³²⁴ in EOC.

1.5 Genetic Variation and Cancer

1.5.1 DNA Sequence Variation

All humans would be a clone if the nucleotide sequences of their genomes were exactly identical. The only known human clones are identical twins. All other humans have nearly 99.9% identical genomic sequence ³²⁵. The remaining 0.1% variation (i.e. one base in every 1000 bases) is medically very important. It is this DNA sequence variation or polymorphism that is responsible for the phenotypic differences among individuals and races e.g. skin colour, facial features, height etc. Even in the same individual,

corresponding loci on homologous chromosomes have variations. The variant DNA sequences on corresponding loci of homologous chromosomes are commonly known as alleles. The most common type of genetic variation is single nucleotide polymorphism (SNP). There are more than ten million SNPs in the human genome. Other less frequent polymorphisms are variable number of tandem repeats (VNTRs), deletion / insertion polymorphisms (DIPs or indels), and inversions. Genetic variations are usually present in the non-coding regions of the genomes / genes. The non-coding regions could be regulatory or of unknown function. However, if SNPs are present in the exons of genes, they may or may not alter the amino acid sequence, and hence function, of the peptide or protein (synonymous or non-synonymous SNPs)³²⁶³²⁷. Increasing evidence suggests that polymorphisms in the flanking regions of genes, the non-coding regulatory regions, influence the level of gene expression, or the location or timing of expression³²⁸.

1.5.2 DNA Sequence Variation and Diseases

An estimated 10,000+ human diseases are known to be monogenic³²⁹. These diseases are rare, highly heritable “Mendelian” disorders in which variation in a single gene is both necessary and sufficient. It is now increasingly apparent that these genes are further modified by other genes known as modifier genes, that cause intra-familial variations, altered penetrance, and altered severity of disease³³⁰. The nature of disease depends on the functions performed by the modified gene. Monogenic diseases result from modifications in a single gene occurring in all cells of the body. Though relatively rare, they affect millions of people worldwide. The single-gene or monogenic diseases can be classified into three main categories:

- Dominant

- Recessive

- X-linked

1.5.3 Gene Polymorphisms and Cancer

Most of the gene polymorphisms identified so far in cancer epidemiology are located in genes encoding for metabolic enzymes involved in phases I and II of chemical metabolism and those responsible for the DNA repair, inflammatory response, cell adhesion and vascular growth³³¹.

Cancers arise from the accumulation of inherited polymorphisms (i.e. SNPs and mutations) and sporadic somatic polymorphisms in cell cycle, DNA repair, and growth signalling genes³³². For instance members of certain ethnic groups have a higher risk of carrying SNPs in cancer genes such as BRCA-1, BRCA-2, or APC (adenomatous polyposis coli). These SNPs confer an increased risk of developing breast, ovarian, prostate, or colon cancers³³³. Somatic polymorphisms such as those in the p53 gene, influence both clinical outcome and response to therapy³³⁴.

1.5.4 The HapMap Project

The identification of the haplotype on which the mutation is located and then subsequent identification of the mutation is a common research approach. This has been shown for several diseases e.g. in cystic fibrosis and diastrophic dysplasia^{335 336}. Sequence analysis of the human genome has yielded numerous SNPs, raising the expectation that new low-penetrance tumour susceptibility genes will be identified that can be used to estimate an individual's cancer risk³³⁷. Several groups have worked to create a SNP map of the whole human genome³³⁸. Notable among these are the U.S. Human Genome Project (HGP) and SNP consortium. The International HapMap Project was launched in October 2002 to create a public, genome-wide database of common human sequence variation, providing information needed as a guide to genetic studies of clinical phenotypes. The HapMap data have generated a genome wide variation resource that will have a huge impact on the investigation of evolutionary forces that have shaped variations in natural populations. The HapMap should be valuable in reducing the number of SNPs required to examine the entire genome for association with a

phenotype from the 10 million SNPs that exist to roughly 500,000 tag SNPs. This will make genome scan approaches to finding regions with genes that affect cancers much more efficient and comprehensive; since effort will not be wasted typing more SNPs than necessary and all regions of the genome can be included.

1.5.5 Early detection of cancers

Cancer is caused by both external factors (chemical compounds, infectious organisms etc.) and internal factors (inherited predispositions and hormones). Some of the factors that lead to development of cancer are avoidable for example smoking, nutritional deficiencies, obesity, and physical inactivity. However, the inherent risks contributed by genetic variation cannot be eliminated and may enhance the effects of prevalent environmental factors thus leading to different outcomes in individuals sharing the same environment. For common cancers such as breast, cervix, prostate, and colorectal cancers, screening examinations are available for early detection but no cost effective screening examinations are available for ovarian cancer. Most of patients are diagnosed at a late stage and hence the 5 year survival rate of ovarian cancer remains poor ³³⁹. A continuing search for biomarkers and risk factors to facilitate early diagnosis is essential to improve the dismal outlook for the patients with ovarian cancers.

1.6 The VEGF Gene and Polymorphisms

Angiogenesis has been established as a major factor in carcinogenesis influencing tumour growth, invasion, and the formation of metastases ^{340 341}. VEGF is believed to play a central role in angiogenesis through a variety of mechanisms, including effects on endothelial cell proliferation, survival and migration³⁴². In ovarian cancer, studies have shown that VEGF is critically involved in various steps of ovarian tumour progression^{286 298 343}.

The gene encoding VEGF is located on chromosome 6 and comprises a 14-kilobase coding region with 8 exons and 7 introns and its coding region spans approximately 14Kb ^{344 345}. The VEGF gene is reported to be regulated by oestrogen, hypoxia, growth factors including epidermal growth factor and cytokines including interleukin 6 ³⁴⁶⁻³⁴⁹. There is considerable variation between individuals in VEGF expression and analysis of the 5' untranslated region of the gene has shown many polymorphisms ^{110 350 351}. The VEGF gene is highly polymorphic with some of the polymorphisms occurring at high frequency. Significant associations have been noted genotype and both VEGF secretion and disease. (Watson CJ 2000, ³⁵²⁻³⁵⁴. At least 30 SNPS in this gene have been described in the literature. The promoter region and the 5' untranslated region of the VEGF gene were first screened for polymorphisms by Watson et al ⁴²⁹. Fifteen polymorphisms were identified, and +405 GG genotype of 405 G/C polymorphism was significantly associated with increased peripheral blood mononuclear cell VEGF protein production.

Several studies have investigated the association of the VEGF gene polymorphisms with diseases in which angiogenesis plays a major role in pathogenesis such as diabetic retinopathy ³⁵², renal cell carcinoma ³⁵⁵, acute renal allograft rejection ³⁵³ and melanoma ³⁵⁶. The polymorphisms described thus far that are associated with an increased VEGF production in vivo, include VEGF 405G/C, VEGF 460C/T, VEGF 936C/T /C and VEGF 1154 G/A.

1.7 CONCLUSION

The high mortality rate of ovarian cancer results mainly from the occult progression of the tumour within the peritoneal cavity, with the initial diagnosis usually only being made at an advanced stage. Understanding the biology

regulation, and implications of the process of invasion and angiogenesis has and will continue to drive new biomarker and therapeutic target identification and intervention. Future studies are needed to determine what biomarkers are predictive of response to therapy or survival in women who receive treatment with an anti-angiogenic treatment.

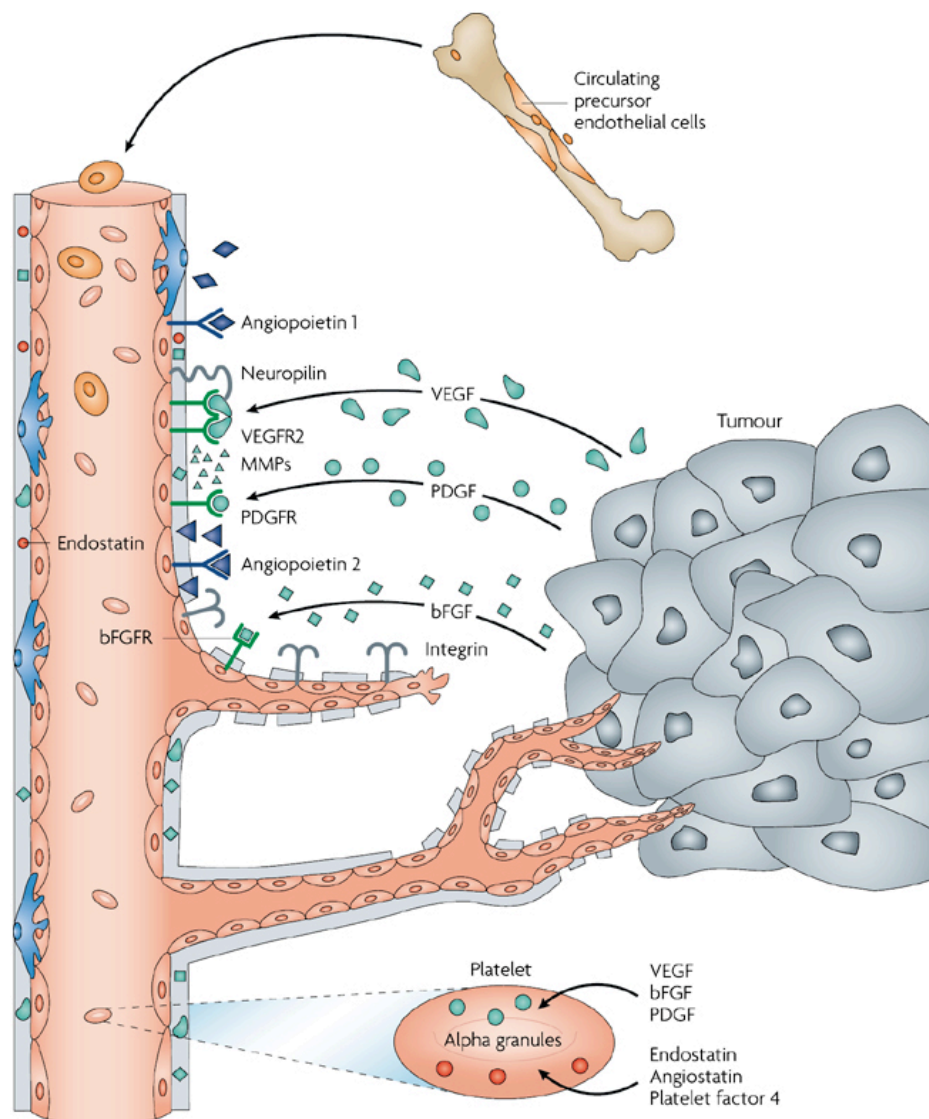


Fig 1.17. Angiopoietin 1 (ANGPT1), expressed by many cells, binds to the endothelial TIE2 (also known as TEK) receptor and helps maintain a normalized state in blood vessels. Vascular endothelial growth factor (VEGF) is secreted by tumour cells and binds to its receptor (VEGFR2) and to neuropilin on endothelial cells. It is the most common of at least six other pro-angiogenic proteins from tumours. Matrix metalloproteinases (MMPs) are released from tumour cells, but also by VEGF-stimulated endothelial cells. MMPs mobilize pro-angiogenic proteins from stroma, but can also cleave endostatin from collagen 18 in the vessel wall and participate in the cleavage of angiostatin from circulating plasminogen.

Tumour cells secrete angiopoietin 2 (ANGPT2), which competes with ANGPT1 for binding to the endothelial TIE2 receptor. ANGPT2 increases the degradation of vascular basement membrane and migration of endothelial cells, therefore facilitating sprout formation. Platelet-derived growth factor (PDGF), an angiogenic protein secreted by some tumours, can up regulate its own receptor (PDGFR) on endothelial cells. Basic fibroblast growth factor (bFGF; also known as FGF2) is secreted by other tumours. Integrins on endothelial cells carry signals in both directions. Integrins facilitate endothelial cell binding to extracellular membranes, a requirement for the cells to maintain viability and responsiveness to growth regulatory proteins. Endothelial cells are among the most anchorage-dependent cells. Certain pro-angiogenic proteins up regulate endothelial integrins and are thought to sustain endothelial cell viability during the intermittent detachments that are required to migrate towards a tumour and to simultaneously increase their sensitivity to growth regulators — both mitogenic (VEGF or bFGF) and anti-mitogenic (endostatin). New endothelial cells do not all originate from neighbouring vessels. A few arrive as precursor bone-marrow-derived endothelial cells. Endothelial growth factors are not all delivered to the local endothelium directly from tumour cells. Some angiogenic regulatory proteins (both pro- and anti-angiogenic) are scavenged by platelets, stored in alpha granules and seem to be released within the tumour vasculature. It was recently discovered that pro- and anti-angiogenic proteins are stored in different sets of alpha granules (depicted in green and red respectively).

Adapted from Folkman J².

1.8 AIMS AND OBJECTIVES

- 1) To examine the role of angiogenesis in EOC
- 2) To examine the association between intra-tumoural VEGF expression as a marker of angiogenesis and correlate its expression with established clinicopathological variables and overall patient survival.
- 3) To understand and illustrate the role of VEGF-A expression and its correlation with disease response to platinum based chemotherapy
- 4) To look at the correlation of a polymorphism in the VEGF gene- VEGF 1154 A/G, known to be associated with the increased production of VEGF protein, to risk of epithelial ovarian cancer in a population based case control study.

CHAPTER 2: MATERIALS & METHODS

2.1 MATERIALS

2.1.1 Reagents

All laboratory reagents are listed along with the manufacturer. The grade of reagents, where applicable, is also given. Molecular biology grade reagents were used in methods involving PCR, e.g. PCR, quantitative real-time RT-PCR and gel electrophoresis.

2.1.2 Clinical samples

The immunohistochemical part of the study was conducted at the Royal Free Hospital and ethical approval from the Local Research Ethics Committee (LREC) was obtained for tissue collection prior to conducting the study. Tissue was removed during routine surgery and placed in formalin and processed routinely through the Department of Histopathology, where the tissue was paraffin wax-embedded, or frozen and stored in the tumour bank. Depending on the availability of the samples, the number examined differed for each part of this study. These samples are described in the respective chapters of the thesis.

2.1.3 Archival tissue

There are a number of methods for fixing tissue to preserve its morphology, depending on the tissue type and which techniques will be used after sectioning. The most widely used fixatives in diagnostic hospital histology laboratories are formalin based, as formalin is a neutral salt employed to maintain tonicity. In this study, either 10% formol saline or 10% neutral buffered formalin (10% w/v formaldehyde in water) was used. After fixation was complete, the fixative was poured off and the tissue was processed using

an enclosed automatic processing system (VIP 2000F/300E) programmed with the following schedule: 10% neutral buffered formalin, 2 hours at 40°C; 70% industrial methylated spirit (IMS), 1 hour at 40°C; 90% IMS, 1 hour, 40°C; absolute IMS, 3 hours, 40°C; xylene, 4 hours, 40°C; paraffin wax, 3 hours 60°C. Tissues were embedded in paraffin wax utilising the Tissue-Tek III. The cast blocks were then left at room temperature to allow the wax to become hard. After the wax hardened, the cast blocks were removed from embedding mould and stored in a dry place at room temperature until required.

The blocks were sectioned at 5µm using a microtome at room temperature. The sections were floated on warmed distilled water (45°C) to prevent creasing, mounted onto 2% 3-aminopropyltriethoxysilane (APES) coated slides (see Appendix I), and dried at 42°C for 30 minutes. The slides were placed in the incubator at 37°C for 2 days to firmly attach the sections to the slides and were stored in a slide box and placed at 4°C until required. Each block also had a corresponding Haematoxylin and Eosin (H&E) stained slide taken for comparison, and histological correlation of tumour and non tumour tissue.

2.1.4 Clinicopathological parameters

The clinico-pathological data such as age at diagnosis, tumour type, grade and stage were obtained from the hospital records.

2.2 METHODS

All procedures were performed using strict laboratory protocols that adhered to Control of Substances Hazardous to Health (COSHH) guidelines (October 1984).

2.2.1 Immunohistochemistry (IHC)

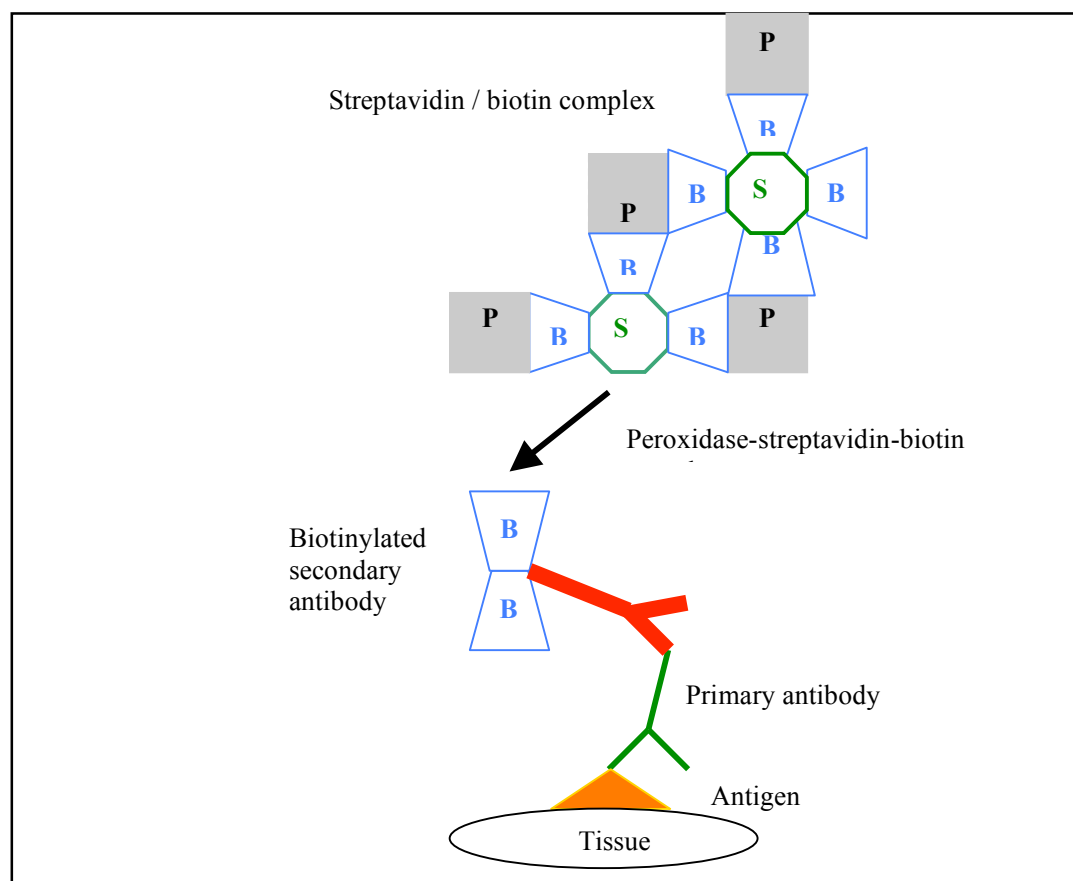
H&E sections were examined by a consultant gynaecological pathologist (JCC) and myself to review the histopathological features.

IHC is the demonstration of antigens in tissue sections by the use of specific antigen-antibody interactions, which culminate in the attachment of a marker to the antigen. Through a series of steps, an enzyme that forms a coloured or fluorescent reaction product (visible with a microscope) is attached to the antibody 'probe,' allowing the presence or absence of the antigen in the cells to be determined as well as enabling an assessment of its distribution and variability within a given specimen to be made (Figure 2.1). This can be accomplished by viewing the stained slide with a standard or specialised microscope. IHC provides a permanent record with excellent preservation of cell morphology and can be performed on formalin-fixed paraffin wax-embedded tissue or on frozen tissue. IHC allows direct visualisation of the cells bearing or lacking the marker of interest, so allowing direct correlation with histopathological features of the diagnostic material.

2.2.2 Immunolabelling with streptavidin-biotin complex

Originally, the glycoprotein avidin was used to conjugate with biotin, however, avidin may bind non-specifically to negatively charged structures such as the nucleus, and as it is a glycoprotein, it can react with molecules such as lectins. In this study, the formation of streptavidin-biotin complex was chosen as the immunolabelling method of highest sensitivity because it uses its high affinity of streptavidin for biotin. Streptavidin is a protein (molecular weight 60 kDa) isolated from the bacterium *Streptomyces avidinii*, and has 4 high affinity binding sites for biotin although not all are used due to molecular orientation. Streptavidin has an isoelectric point close to neutral pH and therefore

possesses few strongly charged groups at the near neutral pH used in IHC detection systems. The physical properties of streptavidin make this protein much more desirable for use in IHC detection, compared to avidin. Streptavidin and biotinylated enzyme are simply mixed at appropriate concentrations and allowed to stand for at least 30 minutes at room temperature for the complex to form. This pre-formed complex is attached to the biotinylated secondary antibody. Careful stoichiometric control ensures that some binding sites remain free to bind with the biotinylated secondary antibody. This allows the pre-formed complex to bind and provides a very high signal at the antigen-binding site (Figure 2.1).



Abbreviations: B-Biotin; S-Streptavidin; P-horseradish peroxidase

Figure 2.1 The Streptavidin-Biotin method for IHC.

2.2.3 Standard IHC protocol

Immunohistochemical analysis for the expression of all the proteins in this work was performed using an indirect peroxidase-based labeling procedure with a streptavidin-biotin-horseradish peroxidase detection system (Dako, U.K, Appendix I). IHC was optimised to ensure reduction of non-specific background staining using a suitable positive control for each respective antibody. For IHC, sections were from formalin-fixed paraffin wax-embedded tissue.

The sections were deparaffinised in xylene and rehydrated in different grades of ethanol up to distilled water. Endogeneous peroxidase activity was quenched with a 3% v/v fresh solution of hydrogen peroxide followed by a wash in phosphate buffered saline (PBS; see Appendix I). To optimise immunoreactivity, antigen retrieval was performed using different methods for each protein:

- **Heat-induced epitope retrieval (HIER)** – this method is used to increase the sensitivity of reactions directed to paraffin resistant antigens, especially those found in the nucleus. The most common HIER methods use pressure cookers, microwave ovens or autoclaves as the heat source and low molarity buffers with acid or alkaline pH^{357, 358, 359, 360}. In this project, several attempts using different conditions such as low to full power of the microwave or pressure cooker, heating incubation time of 2, 5, 10 and 15 minutes and different buffers (sodium citrate or EDTA buffer) were used to enhance the retrieval of the antigens. As a result, microwaving in sodium citrate buffer (pH 6.0, Appendix I) for 10 minutes at full power or pressure cooking in sodium citrate buffer (pH 6.0, Appendix I) for 2 minutes were the established optimum conditions.
- **Protease digestion or protease induced epitope retrieval (PIER)** – Initially, this method was commonly used to counteract the antigen

masking effects of formalin fixation. It involves the use of numerous enzymes such as proteinase K, trypsin, DNase and pepsin which is required for the cleavage of the molecular cross-linking, allowing the epitope to return to its normal configuration, hence enabling more binding of the antibody; however, the cleavage is not specific and some antigens might be negatively affected by this treatment³⁶¹. By trial and error, using different times incubation of 5, 10 and 15 minutes, as well as various concentrations of protease solution of 6, 10, 12.5 and 15 mg, the optimised conditions were found to be the use of protease digestion (Bacterial protease Type 24, Sigma) 12.5 mg in 100 ml of PBS at 37°C for 10 minutes (Appendix I).

- **No antigen retrieval step** – this method is performed for antibodies which are easily detected, without causing any background that may result in non-specific staining.

The sections were then placed in a humidity chamber and a solution of serum (normal rabbit or goat) was added to prevent non-specific staining (10% v/v in PBS, left for 15-30 minutes at room temperature). This serum was then removed by tapping off the excess and the primary antibody was placed on top of the section using the optimum dilution (Table 2.1). All dilutions were performed in bovine serum albumin (BSA) diluted in PBS (see Appendix I) unless specified.

Optimisation of antibody dilutions was performed for all primary antibodies on control tissue sections, initially using the dilution range recommended by the manufacturer and then performing other dilutions to optimise conditions. The sections from the control tissue were then assessed for intensity of staining and absence of non-specific staining. The optimal dilution chosen for each antibody was the one in which there was good quality brown staining which disappeared if the dilution was increased. Incubation times were also optimised for all antibodies, along with the

incubation temperature, allowing specific staining with the lowest concentration of antibody but still providing intense specific staining. Table 2.1 shows the optimum antigen retrieval step used for each protein.

Following incubation, the primary antibody was washed off in two 3-minute washes with PBS. The secondary antibody, was then applied in PBS-BSA containing a 10% dilution of normal human serum (see Appendix I). The secondary antibody uses the primary antibody as its antigen; hence a mouse monoclonal primary would have a secondary antibody raised against the mouse. This secondary antibody had been biotinylated; i.e. biotin had been added chemically. It was added at a dilution of 1:200 and was left at room temperature for 30 minutes. The secondary antibody was then removed by two 3-minute washes with PBS and then reacted with peroxidase-conjugated streptavidin-biotin (Appendix I) at a dilution of 1:200 for 30 min. Excess peroxidase was washed off in 2-3 washes of PBS and peroxidase activity was detected by incubating the sections in a solution of 3, 3'-diaminobenzidine tetra hydrochloride (Appendix I) for 5-8 min until a brown colour had developed. Placing the slides in tap water then terminated the reaction. The slides were counterstained in Mayer's haematoxylin, which is used to define nuclear structures, washed and finally rehydrated with grades of ethanol. They were then cleared in xylene to allow the refractive index to come to 1, mounted in DPX and a cover slip was then added.

2.2.4 Controls

A known positive control was added to each staining batch to ensure that the staining was successful. The same control specimen was used for each run for a given antigen to evaluate the intensity of the stain. The control used was placental tissue in all cases. Negative controls were also included in each

staining run. They were the tissue under investigation with omission of the primary antibody (replaced by PBS) in order to ensure that other reagents do not stain non-specifically. In the negative control for VEGF staining, the primary antibody was replaced by IgG₂B.

To ensure staining consistency and reproducibility, a number of precautions were taken:

- i. Sections from different blocks were stained as a batch on the same day and the process was repeated 3-4 times for each protein to assess the uniformity of the intensity of the staining.
- ii. If available, different blocks (approximately 2-3) from the same specimen were analysed separately and the most representative block was used.
- iii. Results were analysed independently by three observers (Dr Anne Christine Wong te Fong, Dr Julie Crow and me).

In all cases, there was <5% variation in results between sections, blocks and observers. A consensus score was then achieved.

2.2.5 Immunohistochemical staining with VEGF-A antibody.

Sections were deparaffinised in xylene and rehydrated in different percentages of ethanol up to distilled water for 10mins. 3% hydrogen peroxide was placed on the sections to block endogenous peroxidase for 10mins. They were then placed in distilled water for 10min at 37 °C.

Optimisation of the protocol was performed for the primary antibody as above. Optimisation of the antigen retrieval method was performed using 3 different methods; microwaving in sodium citrate buffer(pH 6.0) for 10 minutes at 750watts; protease digestion(bacterial protease Type 24, Sigma) using 12.5mg in 100ml of PBS at 37C for 10mins, and finally using no antigen retrieval. Different protease digestion times (using different time intervals of 5,

7.5, 10 and 15mins) and protease concentrations (6, 10, 12.5 and 15mg) as well as different incubation times and temperatures were also tried. The optimal incubation time and temperature was identified when specific staining was achieved with the lowest concentration of antibody but still providing intense staining.

Sections were stained on three separate occasions to ensure reproducibility. Results were analysed by three independent observers (GKS, JCC, LFWTF). In all cases there was < 5% variation in results sections and observers.

Table 2.1 : Antibodies and Optimum conditions used for Immunohistochemistry.

Antigen	Source	Clone	Working dilution	Antigen Retrieval	Positive control	Negative control	Incubation time	Incubation temperature
Human VEGF	R&D Systems, Abington, UK	26503.1 IgG2B	1:40	Protease digestion	Placenta	IgG2B	Overnight	4°C

Biotinylated rabbit antimouse serum (Dao, Cambs, UK) dilution 1: 400 in TBS, PBS was used as the secondary antibody in all cases.

2.2.6 VEGF Quantification

For the assessment of VEGF, regions of interest containing tumour cells and no connective tissue stroma were defined by two independent observers (JCC & GKS). Each slide that contained tumour was analysed by using a eye-piece graticule that defined an area of 1cm x 1cm at a magnification of 200 times (*i.e* with x20 objective). That graticule comprised 100 individual squares (each 1mm x 1mm) and was placed over all fields of the slide that contained tumour (minus large areas of connective tissue), All areas that contained tumour were analysed. The identification of at least 1 VEGF-positive cell within the confines

of an individual square defined that square as a positive focal area. The number of VEGF-positive focal areas was added up for each slide and then an average number of focal areas for that case were obtained. Using the tariff shown in Table 1 (scoring system devised by GKS and JCC), the average number of focal areas per case was converted into a score for VEGF, on an increasing linear scale of 0 – 5, (where 0 = 1 - 4 focal areas, and 5 >50 focal areas).

Table 2.2. VEGF score by IHC as a function of the number of focally positive VEGF areas

	Number of focal areas per cm ² of tissue section										
	1-4	5-9	10-14	15-19	20-24	25-20	30-34	35-39	40-44	45-50	>50
Score	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0

2.2.7 Statistical Analysis

The correlation between VEGF-A levels and other prognostic variables was statistically analysed by means of Pearson's chi squared test, Spearman rank correlation analysis and univariate analysis. Survival rates were examined using Kaplan-Meier plots of analysis. The statistical significance of differences between the survival rates of groups with different VEGF expression ie high or low expression was assessed by the log-rank test. The independent prognostic significance of variables was assessed by multivariate analysis, by means of a multivariate Cox regression model. P values of <0.05 were considered statistically significant. All statistical analysis was carried out using SPSS version 15.0.

Expression of the intratumoural VEGF-A protein was correlated with platinum sensitivity (using Mann-Whitney U Test and a Fisher's Exact Test). Spearman Rank correlations were calculated to test for association between VEGF-A expression and, patient age, tumour stage, grade and survival. Survival times were estimated in months from the date of operation to the date of death (or date of last follow-up). Kaplan Meier survival plots and a log rank test were used for the univariate analysis. The Cox Proportional Hazard Model was used for multivariate analysis.

Univariate and Multivariate Logistic regression was used to investigate the association with clinical response to platinum based chemotherapy of each of the variables described above. Response to therapy was defined as for the 2003 revision of the 'Gynaecologic Oncology Group' criteria.³⁶² Patients who had progressed on chemotherapy or who relapsed within 6 months of completing of completing their primary therapy were defined as platinum resistant. All other patients were categorised as responders to platinum-based therapy. Recurrence of tumour was defined by imaging modalities (MRI and MRI modalities) and/or by the demonstration of increasing levels of CA-125 in the serum.

Patients were divided into two groups based upon the intra-tumoural VEGF expression, the median value of the VEGF score, which was 3, was taken as the cut off point. Therefore the groups were:

- 1) High VEGF expression; score of 3 or > 3.
- 2) Low VEGF expression score of <3.

These two data sets were used to analyze the data statistically.

A cut-off point for scoring of VEGF as high expression (≥ 3) and low expression (< 3) was used to analyse some of the data statistically.

2.3 CASE CONTROL STUDY

2.3.1 Study Design

Genetic studies for common diseases fall into many broad categories: family-based linkage studies across the entire genome, linkage disequilibrium mapping studies, and population-based association studies of individual candidate genes. Genetic association studies in cancer are complex because of the multi-stage process of cancer and difficulties in analysing genetic variants in population and family studies. This study fell under the last category and the basic question this study was designed to answer was “whether the suspected genetic factor was associated with the outcome of interest ie development of cancer?” Prospective studies are considered to be the most powerful and reliable because by randomisation they are able to balance the unknown prognostic factors. However, since ovarian cancer is comparatively a less frequent cancer and given the time and logistical constraints of this study, it was not possible in this study setting to achieve a sufficient sample size or proper follow up required in a prospective study. A case-control study was therefore designed to assess the relationship between the described genotype and the presence of cancer, the outcome event. The exposure of primary interest was the presence or absence of the genotypes or haplotypes determined by the chosen set of angiogenesis related genes. The outcome events in this study were the development of epithelial type of ovarian cancer. The objective was to compare exposure history between the case and the control groups.

The strengths of this case-control study were:

- It was less constrained by the natural frequency of the disease.
- It greatly shortened the waiting time generally required by cohort studies.
- It was low cost and it had fewer practical problems.
- It was ideal for the assessment of the chosen disease aetiology.

- The chosen population are known to have a high rate of consanguinity; therefore the sample size could be smaller.

The drawbacks of this retrospective case-control study were:

- Randomisation was not possible and population at risk was undefined
- Selection biases were unavoidable and causal interpretation was unattainable due to presence of confounding factors
- Since a baseline had not been defined there was no absolute measure of many confounding variables.

2.3.2 Target population

The aim of the study was to obtain the information about and apply the result to the Pakistani population. For both the study groups (cases and controls) the target population was individuals of Pakistani ancestry who had been resident in Pakistan for most of their lives.

2.3.3 Patients

This case-control study was undertaken in a population based ovarian cancer collection from four tertiary referral centres and hospitals in Karachi (Liaquat National Hospital, Aga Khan University Hospital, and Ziauddin University Hospital) and Lahore (Shaukat Khanum Memorial Cancer Hospital and Research Centre). These centres were selected, due to the fact that most of the ovarian cancer patients were known to attend the centres selected. The study was conducted between September 2005 and June 2007. A total of 400 cases of epithelial ovarian cancer (EOC) were identified and recruited from the above named centres. During the same period 438 controls were recruited and interviewed. The controls consisted of women attending hospital based outpatient clinics.

2.3.4 Case definition

A case was defined as:

An individual of Pakistani ancestry diagnosed with histologically proven epithelial ovarian cancer and undergoing treatment at any four of the participating centres as mentioned previously and having relevant patient information available in the hospital records either in electronic or paper form in the Department of Oncology and/or Department of Obstetrics and Gynaecology.

2.3.5 Outcome of Interest

Primary Epithelial Ovarian Cancer

2.3.6 Bias considerations

In designing the study the following sources of bias were considered.

- Recall bias: It did not affect this study because the main variable under study did not depend upon the memory of the cases under study. Most of the data were obtained from the epidemiological questionnaire
- Confounding bias: This is an inherent bias of case-control study, and could not be avoided because ovarian cancer patients are diagnosed at a late stage in life when co-morbidities are generally present, and there is no available technique at present to measure the stepwise DNA damage that occurs with advancing age. However, care was taken at analysis stage to handle this issue wherever possible.
- Berkson's Bias: A Berkson's bias is introduced if subjects who are both exposed and diseased are more likely to be admitted to the hospital. The proportion of exposed in the disease groups can be inflated artificially. E.g. in a study for an association between low birth weight

and cerebral palsy, infants having both low birth weight plus palsy are more likely to be admitted as compared with cerebral palsy alone. This would distort the real effect of exposure when cases are selected only from the hospital. Berkson's bias did not affect this study because the exposure under study did not by itself increase the chances of admission to a tertiary care hospital.

- **Neyman's Bias:** A Neyman's bias is introduced when the study base does not capture all the cases it is supposed to capture, thus distorts the true prevalence of the disease e.g. if the disease is very transient (or leads to early death) or this could also happen to studies of asymptomatic diseases (HPV). Neyman's bias could not be avoided although the study was done in four centres and missed the cases that were representative of other centres. However, the results of the study were not affected by this bias because an exact measure of prevalence was not required.

2.3.7 Selection of Controls

The controls consisted of women attending hospital based outpatient clinics. Hospital matched controls were selected to balance the hospital related confounding factors, additionally it helps in excluding the possibility that the exposure itself is a cause of admission to hospital (please refer to definition of Berkson's bias in previous section). It also reduces the cost of the study. However, the inferences drawn from the hospital controls would not be valid if the distribution of exposures are significantly different from population controls. The controls in this study were selected from the fracture clinics and obstetric and gynaecology outpatient clinics. The inclusion criteria for controls were women not to have a personal or family history of ovarian or breast cancer.

2.3.8 Study setting

The study was part of the Pakistani Ovarian Cancer Study carried out in the departments of oncology and Obstetrics and Gynaecology at four tertiary referral centres in Pakistan, in two cities in Pakistan, Lahore and Karachi. The participating centres although tertiary centres covered a wide geographical area and had a catchment population that covered all socioeconomic and ethnic groups, within the Pakistani population.

2.3.9 Inclusion Criteria

- Proven cases of primary epithelial ovarian cancer
- Pakistani ancestry for both cases and controls
- Availability of medical records of cases
- Controls free from any malignancies both without personal history and family history.

2.3.10 Exclusion Criteria

- Presence of DNA from tumour tissue
- Personal or family history of breast or ovarian cancer in the controls
- Non amplification in PCR
- Non-reproducibility of genotyping result

2.3.11 List of Variables

2.3.11.1 Dependent Variables

- Tumour histology: All cases were epithelial ovarian cancer.
- Post operative outcome: This was measured from the date of operation to death or recurrence of the disease.

2.3.11.2 Independent Variables

2.3.11.3 Variables of Primary Interest

- VEGF 1154 A/G polymorphism; (SNP rs ID 3024997 number; Individual sequences can be referred to by their refSNP cluster ID numbers).
- Postoperative outcome

2.3.11.4 Other Variables

- Age
- Tumour size: This was both of the primary and metastatic tumour.
- Tumour Grade
- Tumour stage: based on the International Histological Classification of Ovarian Tumours recommended by FIGO.

2.3.12 Sample Size Calculation

The aim of sample size calculation for this study was to enable a judgement that is accurate and reliable, to get a precise estimate of proportions in the two groups and at the same time not waste resources and time by choosing a larger than required sample size. The sample size calculation related to final achieved sample allowing for response rates and loss to follow up. The calculation was based on a power of 80%, an effect size of 20 percent.

Since this is the first study in the Pakistani population that undertook to examine the distribution of genotypes in ovarian cancer patients, no previous data were available to presume a proportion of outcome in a particular genotype or haplotype or *vice versa*. For the purpose of calculating sample

size proportions p_1 and p_2 of 60% and 40%, respectively, were assumed and an effect size of 20 % was taken as clinically significant difference in outcomes (<http://www.sgul.ac.uk/index.cfm?DD25D103-C079-6609-F78D-BC005970DCD9>).

$$n = \frac{[A + B] \times [\langle p_1 \times (1 - p_1) \rangle + \langle p_2 \times (1 - p_2) \rangle]}{[p_1 - p_2]}$$

n = required sample size for each group

A = depends on desired significance level. 1.96 for 5% significance level

B = depends on desired power. 0.84 for a power of 80%.

p_1 = High risk allele frequency in cases (primary outcome variable in this study was either presence or absence of ovarian cancer)

p_2 = High risk allele frequency in controls.

$$n = \frac{[1.96 + 0.84] \times [\langle 0.6 \times (1 - 0.6) \rangle + \langle 0.4 \times (1 - 0.4) \rangle]}{[0.6 - 0.4]}$$

n = 294 in each group

2.3.13 Ethical Considerations

In designing this study, it was recognised that genetic susceptibility biomarkers could have an impact not only on the study subjects but also on their families. Biomarkers that are obtained from the individual person have the potential for providing important information about exposures, biological effects of exposures, and susceptibility for disease for that individual and her family. No medical interpretation of the genotyping result was provided to the patients or any other individual. However, it is recognised that study subjects have rights to appropriate information before, during and after the study. It was explained to the participants of this study that the results obtained were part of a research study, and the clinical interpretation and importance of the

presence of the polymorphism in question was not known and that any results would have to be verified in a larger population based study.

Ethical approval was obtained from the Ethics committee of each individual participating centre prior to the start of the study. The individual hospital based Committees operate according to the general principles of medical ethics including the Declaration of Helsinki.

Written assurance was provided to the Ethical committee in each individual institution that the leftover genetic material would not be used in any other study without fulfilling legal and ethical requirements. The stored genetic and biological material is neither identifiable nor traceable back to the donor. Care was taken to inform the participants of their freedom to refuse participation in the study. The information leaflet, was translated into the local language Urdu, and was provided to each participant and given enough opportunity to carefully peruse the leaflet. Informed consent form was approved by the ethical committee. Written informed consent was obtained from participants. Use of patient's records/histological records has been explained elsewhere in the material and methods section. Confidentiality of data was ensured and access was limited to directly concerned researchers only. The data were stored and reported in a way that did not identify the study participants to a third party. After obtaining relevant information about the study subjects they were coded and anonymised and could not be traced back to subject identification.

2.3.14 Data collection

A comprehensive clinicopathological database of all patients was constructed in an Obstetric and Gynaecology Department of each institution, clinical information from the patients' charts was entered into the electronic database by the research officer assigned to the study at each centre.

Genotyping and haplotyping data were entered into SPSS, as and when it became available, throughout the duration of the study.

2.3.15 Sample collection and storage

Data collection and sample collection was started in September of 2005 and continued up to June 2007. This study was running in parallel with the Pakistani Ovarian Cancer Study (POCS), which is looking at the epidemiological factors associated with ovarian cancer in the Pakistani population. To date this study has over 2000 ovarian cancer cases and 3000 controls, with both DNA from study subjects and epidemiological data. This has been collected over a period of 5years and is part of the International Ovarian Cancer Consortium, examining genetic and lifestyle factors predisposing to the development of ovarian cancer.

The blood samples for both cases and controls were collected by the research officers assigned to the study at each participating centre. A total of 10 ml of venous blood was drawn in a purple top tube (vacutainer) containing EDTA (ethylenediaminetetraacetic acid) as an anticoagulant. All tubes were labelled with a sample ID. Blood samples were either refrigerated or frozen until extraction of DNA.

Patient names and other information linking the samples to the patients' identification were removed and replaced by sample ID numbers. The researchers involved in the PCR analysis of these samples were blinded to clinicopathological data, of the samples.

2.3.16 Selection of polymorphism in the VEGF gene and Primer Design

2.3.16.1 DNA extraction

1. REQUIREMENTS

a. CONSUMABLES

1. 50 ml polypropylene centrifuge tube
2. 15 ml polypropylene centrifuge tube
3. 1.5 ml centrifuge tube
4. Blue (100-1000 microlitres) and yellow (10-200 microlitres) tips for pipettes.

b. REAGENTS

1. Solution A
2. Solution B
3. Sodium per chlorate (5M)
4. Chloroform
5. Absolute Ethanol / Isopropanol
6. 70% Ethanol
7. Autoclaved-deionized water / TE (Tris EDTA)

c. EQUIPMENT

- Water Bath
- Centrifuge
- Micro Centrifuge
- Vortex
- Incubator
- Refrigerator

d. RECIPES OF SOLUTIONS

SOLUTION A (1 Litre)

Tris = 1.21 gm
Sucrose = 109.5 gm
MgCl₂ = 1.02 gm (Hexahydrate)
Triton (100%) = 10 ml
Adjust the pH at 8
Autoclave

SOLUTION B (1 Litre)

Tris = 48.5 gm
EDTA = 22.3 gm
NaCl = 8.77 gm
Autoclave
Add 10 gm SDS

2. PROCEDURE

i. PREPARATION

- Prepare solution A and solution B.
- Set a water bath at 65°C.
- Label a 50 ml polypropylene centrifuge tube.
- Add 5-10 ml of blood to the tube.
- Fill the tube with 40 ml of solution A.
- Spin for 20 minutes at 2500 rpm.
- Dump the blood out into the appropriate waste container, leaving behind a cell pellet.

ii. CELL Lyses

- Add 2 ml of solution B and vortex briefly to re-suspend the cell pellet.

iii. DEPROTEINISATION

- Add 500 µl of Sodium per chlorate (5M) to the cell suspension and mix manually.
- Incubate the tubes in a 65°C water bath for 25 minutes.

iv. DNA EXTRACTION

- Add 2 ml of Chloroform to the cell suspension, vortex and spin at 2500 rpm for 15 minutes.
- Pipette out the top (DNA) layer into a fresh 15 ml centrifuge tube.

v. DNA PRECIPITATION

- Add 2 volumes of Absolute Ethanol / Isopropanol.
- Invert gently to precipitate the DNA.
- Keep the tube inverted for 5 minutes.
- When DNA is settled at bottom then decant Isopropanol.
- Add 1 ml of 70% Ethanol.
- Transfer it in 1.5 ml centrifuge tube.
- Spin Eppendorf tubes at 10,000 rpm for 15 minutes.
- Decant 70% Ethanol and allow DNA to air dry to remove excess alcohol.

vi. DNA RESUSPENSION

- Re-suspend the DNA in water / TE (Tris EDTA).
- Incubate it at 37°C for 12 hours.
- Then place at 4°C for storage.

vii. READ O.D AT RATIO 260/280 nm BY SPECTROPHOTOMETER

- First make dilution (100 percent) of dissolved DNA sample.
- Fill 1.5 ml centrifuge tube with 495 ul Autoclaved deionized water.
- Add 5 ul DNA into above tube, mix it and then take O.D.
- Note down the quantity and quality of the DNA.

2.3.16.2 Selection of Polymorphism in VEGF Gene, Primer Design and SNP Genotyping

Tetra-Primer ARMS-PCR

It is known that single nucleotide polymorphisms (SNP'S) occur approximately once per 250-1000bp and account for approximately ~90% of DNA sequence variation in the human genetic structure ³⁶³. Analysis of SNP'S has been increasingly used in studying genetic determinants of complex diseases. The stability and large numbers of SNPs within the genome, make them especially useful DNA markers ³⁶⁴.

Population genetics and genetics association studies require the analysis of large numbers of subjects, it would be beneficial if techniques were employed for SNP genotyping that were, simple, low cost, and high throughput, such as Tetra-Primer ARMS-PCR. This technique was chosen for this study, as it is known to be simple and economical, especially relevant for research in low resource setting such as Pakistan. It involves employing two primer pairs to amplify, respectively, the two different alleles of a SNP, in a single PCR reaction, followed by gel electrophoresis, it utilises some of the certain principles of the tetra-primer PCR method ³⁶⁵, and the amplification refractory mutation system (ARMS) ³⁶⁶. The main advantage of this method is that it only requires a single PCR reaction and does not need post PCR manipulation, making it rapid, simple and cheap. The initial planning of primers, is a critical step of this technique but is often time consuming. To circumvent this problem a primer design computer programme, which has made this task automated and accessible through the Internet, at <http://cedar.genetics.soton.ac.uk/-publiic/html/primer1.html>. Users need to input the target DNA sequence, specify the polymorphic site and define criteria for the primers (Tm, %GC, length and complementarities) and product sizes ³⁶⁵.

Single nucleotide polymorphism (SNP) was selected in the VEGF gene (Table 2.3). This polymorphism is non coding and is intronic. Flanking sequence was retrieved from ENSEMBL. The tetra primers ARMS-PCR procedure³⁶⁵ was used to genotype the SNP (rs3024997). The method employs four primers to

amplify a larger fragment from DNA containing the SNP and amplicons representing each of the two allelic forms (Table 2.3). Primers were designed using web based software made accessible by Ye.et.al (http://cedar.genetics.soton.ac.uk/public_html/primer1.html).

Optimisation of tetra-primer ARMS PCR for detection of rs3024997 polymorphism was performed empirically. For outer-inner primer pair Mg²⁺ concentrations was tested from 1.5 mM to 4 mM. Searching for the optimal one, annealing temperatures were tested from 53°C to 67°C by using the gradient function of thermal cycler (Eppendorf). The usually suggested outer–inner primer ratio (1:10) for tetra-primer ARMS PCR (Ye.et al 2001) was used. Finally optimised PCR was performed in a total volume of 10 µl containing 200-300 ng of template DNA, 1 pmol of each inner primer, 0.1 pmol of each outer primers, 200 µM dNTP, 2 mM MgCl₂, 1Xbuffer and 0.5 U of Taq DNA Polymerase (Promega).

PCR conditions were: initial denaturation at 94°C for 7 minutes followed by 34 amplification cycles each consisting of denaturation at 94°C for 45 seconds annealing at -66.2°C for 45 seconds and extension at 72°C for 45 seconds and final extension at 72°C for 7 min.

Agarose gel electrophoresis was used to separate and analyse DNA. The DNA was visualised in the gel by addition of ethidium bromide (EtBr). EtBr binds strongly to DNA by intercalating between the bases and is fluorescent. 2% agarose gels were made in all cases. Different sizes of gels (with varying numbers of wells) and gel tanks were used. 10 µl of PCR product along with 2 µl of loading dye was used to load the wells. Since the size of the DNA fragments ranged between 100 and 500 bps, a 100 bp ladder was used to identify the sizes of fragments.

The amplified products were electrophoresed in a 2.5 % agarose gels made by adding 2.5gm of agarose to 100ml of 0.5X TBE at pH 8, the solution was then carefully brought to boil in a microwave oven to dissolve agarose. 0.7 ul of ethidium bromide (stock 10mg/ml) was added and solution was stirred to

disperse ethidium bromide and then poured into the gel casting tray . Combs were inserted into the gel and were taken out on solidification of the gel. The gel together with the tray was put in an electrophoresis chamber and TBE of same concentration was poured to completely cover the gel. 10ul of amplified products were loaded in to the wells, the lid of the electrophoresis chamber was closed and current (100 V) for 45 minutes was applied. DNA moved towards the anode due to negative charges on its phosphate backbone. After 45 minutes gel was visualised under UV trans-illumination (GelDoc®, Biorad, Hercules, Ca, USA).

Table 2.3.SNP ID and Primer Sequences

refSNP ID	Polymorphism	Primer Sequence(5'-3')	Tm (°C)	Amplicon size (bp)
rs3024997	G/A	Forward inner primer(A allele): GATTTTGGGAAGGACTTGCCTGATGCA	63.2	208(A allele)
		Reverse inner primer(G allele): CTGTAATGCCACTCTTTGGAGCTGCC	66.4	144(G allele)
		Forward outer primer: GTGTGATCTCTGGAATGAAAACAGGCC	65.1	300(from two outer primers)
		Reverse outer primer: ATCAGGGTACTCCTGGAAGATGTCCAC	68.0	

2.3.17 Statistical Analysis

The statistical analysis was carried out by Professor Philippe Frossard and Dr Danish Salaheen, at the Centre for Non Communicable Diseases Pakistan and Strangeways Laboratory Cambridge University UK.

2.3.7.1 Statistical software and Data entry

STATA version 10 was used for all data entry purposes. The original data were obtained in Excel format. The data were transferred to STATA data

editor from excel and variables were labelled in the variable view of the STATA data editor.

2.3.17.2 Descriptive Statistics

Tables were made using C tables in SPSS. Summary of data e.g frequency counts, percentages, was obtained using the descriptive and graphs menu in SPSS. Means, Medians, Standard deviations, and Ranges were given in case of continuous variables. Characteristics of groups have been described in appropriate sections.

2.3.17.3 Data Analysis

The association between VEGF rs 1570360 and EOC was modelled through multivariate logistic regression analysis. Odd ratios and confidence intervals were used to assess the occurrence of VEGF -1154G>A genotypes in patients with EOC cases compared with the control group. Significance testing was carried out by combining chi-square tests and then comparing the two independent proportions. Adjusted OR's for the epidemiological covariant such as history of gynaecological cancer and/or other cancer types in the family, tumour stage and grade, age and ethnicity, were determined by using a multivariate logistic regression method. Survival associated with each of the three genotypes was estimated by Kaplan-Meier survival analysis. We considered results with $P < 0.05$ as statistically significant. STATA 10.0 was used to carry out the statistical analyses.

The measure of Hardy-Weinberg disequilibrium was calculated as the function of the difference between the observed and expected genotype frequencies. Estimation of departure from Hardy-Weinberg proportions was evaluated with the help of D_A statistic and tests of statistical significance were applied as reported by Haviland.³⁶⁷

CHAPTER 3: PROGNOSTIC SIGNIFICANCE OF INTRA-TUMOURAL VEGF AS A MARKER OF TUMOUR ANGIOGENESIS IN EPITHELIAL OVARIAN CANCER

Publication:

Prognostic significance of intra-tumoral Vascular Endothelial Growth Factor (VEGF) as a marker of tumour angiogenesis in epithelial ovarian cancer. Siddiqui GK, Elmasry K, Wong Te Fong AC, Perrett C, Morris R, Crow JC, Maclean AB. *Eur J. Gynecol Oncology* 2010; 3(2): 156-9.

3.1 INTRODUCTION

3.1.1 ANGIOGENESIS

Angiogenesis is the formation of new capillary blood vessels from pre existing microvessels. Vasculogenesis is the process whereby the early vascular plexus forms from the mesoderm by the differentiation of angioblasts (vascular endothelial cells that have not yet formed a lumen), which subsequently generate primitive blood vessels. Mesoderm-inducing factors of the fibroblast growth factor are crucial in inducing paraxial and lateral plate mesoderm to form angioblasts and haematopoietic cells. Angiogenesis precedes and sustains tissue growth and therefore plays a critical role in normal tissue physiological processes such as reproduction, wound healing or bone remodelling^{368 369}.

Angiogenesis involves the migration of endothelial cells from the parent vessel towards a chemo-attractant. As well as the ability to promote endothelial cell migration, angiogenic factors must also degrade the matrix through which the cells move. Endothelial cell invasion, as part of the angiogenic process, involves secretion of urokinase-type plasminogen activator and its inhibitor (PAI-1). Fine maintenance of the proteolysis balance seems to be crucial for the correct development of new blood vessels. Having migrated, the endothelial cells must proliferate, lay down their own basement membrane and form a lumen, which is the basis of the new capillary. This sequence of events: cell migration, matrix degradation and proliferation, is the basis of angiogenesis. Many different factors have been identified that promote angiogenesis; the most extensively studied is vascular endothelial growth factor (VEGF)³⁷⁰.

3.1.1.1 TUMOUR ANGIOGENESIS and its role in ovarian cancer

It is recognised that tumour angiogenesis is critical for tumour growth beyond 2mm and is associated with prognostic significance in a variety of solid malignancies such as breast, prostate, gastric and non–small cell lung cancer^{129 371}.

In theory, the development of tumours is associated with two phases of tumour growth^{8 97 372}. First is the pre vascular phase which is seen with various intraepithelial neoplastic lesions, can persist for many years and are not capable of metastasis⁹⁶. In contrast the “vascular phase” is characterised by rapid tumour growth and potential for metastasis. However results from studies in EOC are conflicting and the clinicopathological significance has been debated^{129 315 316 373}. Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein expressed in a wide variety of normal and transformed cell types⁵⁵. VEGF is a key angiogenic factor and acts as a highly specific mitogen promoting endothelial cell migration and inhibiting apoptosis¹². Studies have suggested a specific role for VEGF in various phases of ovarian carcinogenesis with effects on tumour growth and neovascularisation illustrated in animal models and in humans^{298 343}. Angiogenesis was suggested as a prognostic factor in various solid tumours but its clinicopathological significance in ovarian cancer and its exact role is unclear. In xenograft models of human EOC, the expression of VEGF is associated with the formation of ascites and the tumour growth rate is proportional to the Microvessel density MVD, which correlates with VEGF, and indirectly with angiogenesis²⁹⁷.

Previous research has shown an association with the high expression of VEGF in EOC and worse overall prognosis in EOC and others have attempted to explore the role of VEGF on the development and progression of EOC²⁸⁹.

3.1.1.2 VEGF

Vascular Endothelial Growth Factor (VEGF), a heparin binding glycoprotein was originally identified in the media conditioned by normal bovine pituitary folliculostellate cells¹⁰ and by a variety of transformed cell lines^{11 13 374}. It is a multifunctional cytokine with potent angiogenic activity. VEGF stimulates angiogenesis through its action as an endothelial mitogen and its ability to increase vascular permeability.

The VEGF family belongs to the platelet-derived growth factor (PDGF)/ VEGF supergene family. Having a homodimeric structure with 8 conserved cytokine residues in a monomer peptide. The VEGF family has at least 7 members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, PlGF and snake venom-derived VEGFs. VEGF-A is a key player in vasculogenesis, as well as angiogenesis^{21 22 375}.

VEGF-A binds and activates VEGFR1 and VEGFR2 but not VEGFR3. The major subtypes of VEGF-A in humans are the 121, 165, and 189-amino acid types (18). VEGF-A165 is the dominant subtype protein among VEGF-A proteins in terms of amount and biological activity. VEGF-A165 binds with neuropilin-1 (Nrp1) and heparin, which contributes to a gradient of VEGF-A165 surrounding VEGF-A expressing cells. This gradient is known to be important for the stimulation of vascular endothelial cells, and for strong angiogenesis²³.

The biological effects of VEGF are mediated through the activation of specific tyrosine kinase receptors expressed mainly on angioblasts and endothelial cells³⁴. VEGF acts selectively on endothelial cells by binding to specific class III receptor kinases. The receptor VEGFR-2 binds VEGF, VEGF-C and VEGF-D and promotes the proliferation and motility of endothelial cells. VEGFR-1 shows a 10fold lower kinase activity than VEGFR-2 and has been proposed to act as a negative regulator of VEGF-induced endothelial cell proliferation³⁷⁶; VEGFR-3 which in adults is exclusively expressed in lymph node endothelial cells, binds VEGF-C and VEGF-D³⁴.

Anti-VEGF therapy has been used in clinical trials to inhibit angiogenesis and is showing promising results in cancer therapies. Bevacizumab is a humanized recombinant antibody that binds to all isoforms of VEGF-A and

inhibits VEGF-A induced endothelial cell proliferation in vitro models¹¹¹. It has shown promising results in several non-gynaecological tumours, especially in colorectal cancers³⁷⁷. Its application in the treatment of ovarian cancer is currently being tested³⁹.

3.1.2 Prognostic Factors in Epithelial Ovarian Cancer

Surgery accurately stages the disease and allows the evaluation of a series of clinicopathological variables that are predictive of the prognosis of the patient and are often used to guide and select postoperative therapy. Established prognostic factors include:

- Tumour Stage and Distribution
- Volume of Residual Disease
- Histological Subtype and Grade.

3.2 AIMS

Our study aims to determine intra-tumoural VEGF status in patients with EOC, and to investigate its relation to prognosis. The elucidation of the prognostic role of VEGF in patients with EOC may enable us to stratify patients with EOC into groups based upon the expression of VEGF, and to tailor treatment schedules, developing novel therapies to inhibit angiogenesis. These therapies are more likely to be most effective in tumours expressing high levels of VEGF.

3.3 MATERIALS AND METHODS

3.3.1 Patients

VEGF expression was studied in 105 primary EOC samples, in patients who underwent surgical staging and tumour debulking at the Royal Free NHS Trust, London, UK between 1995 and 2000. Information on tumour grade, stage, presence or absence of residual disease after staging, and age at diagnosis was extracted from the case notes. Only patients considered to have primary EOC were included in this study. Survival was calculated from the operation date until death or December 2005 when any survivors were censored.

3.3.2 Sample Collection and Preparation

Clinical data and patient information were collected from the patient's clinical records. Ethical approval was obtained for this retrospective analysis from the Royal Free Hospital and Medical School Research Ethics Committee (code number 09/H0720/87). Informed consent was obtained as per local guidelines.

3.3.3 Tissue Specimens

One hundred and five specimens were retrieved from the Histopathology department at the Royal Free Hospital UK. The archival cases had all been formalin fixed and paraffin embedded. In patients who underwent surgical staging and tumour debulking for primary epithelial ovarian carcinoma at the Royal Free NHS Trust London, between 1995 and 2000. Stage was

determined according to International Federation of Gynecology and Obstetrics (FIGO 1986). Only patients considered to have primary EOC were included in the study.

A single consultant histopathologist (JCC) at the Royal Free Hospital originally diagnosed all cases. JCC reviewed all of the haematoxylin and eosin (H&E) stained slides of the cases to confirm the original diagnosis. Wax embedded sections of ovarian and omental tissues, 5µm thick were cut using a microtome and mounted on 3-aminopropyltriethoxysilane (APES) coated glass slides to aid adherence. Slides were stored at 4°C if they not used immediately as there have been reports of loss of antigen when left at room temperature. These were placed onto 2% 3-aminopropyl-triethoxysilane (APES) coated slides (see Appendix I), and dried at 42°C for 30 minutes. The slides were placed in the incubator at 37°C for 2 days to firmly attach the sections to the slides and were stored in a slide box and placed at 4°C until required. Each block also had a corresponding Haematoxylin and Eosin (H&E) stained slide taken for comparison.

3.3.4 Optimisation of the immunohistochemical protocol

Immunohistochemistry was optimised to ensure reduction of non-specific background staining, using suitable positive controls for each respective antibody.

Optimising of antibody dilutions was performed for the primary antibody starting with the recommended manufacturer's dilution and then using a range either side of this. Incubation time was also optimised for the antibody along with incubation temperature allowing for specific staining with the lowest concentration of antibody, but still providing intense specific staining.

Various antigen retrieval steps e.g. none, microwaving, pressure-cooking and protease digestion were also optimised using various concentrations and

incubation times. A known positive control was used in each staining procedure to ensure optimal quality of reagents and methods and to ensure that restaining was successful.

The same control specimen was used for each run for a given antigen to assess the intensity of the stain. Negative controls (same specimen as positive control) were also included in each staining run (Table 3). In the negative controls the primary antibody was omitted and replaced by Phosphate Buffered Saline (PBS) in one reaction, and the secondary antibody replaced by PBS in another.

3.3.5 Specific Immunohistochemical Staining with VEGF-A Antibodies

See Materials and Method Section 2.25 p86-87. Tissue sections were deparaffinised in xylene (3x5min) and then rehydrated in different percentages of ethanol up to distilled water (3x5min). 3% hydrogen peroxide was placed on the sections to block endogenous peroxidase for 10mins. They were then placed in distilled water for 10min at 37 °C.

3.3.6 VEGF Quantification

See Materials and Methods Section 2.26 p87-88

Table 3.1 VEGF score by IHC as a function of the number of focally positive VEGF areas

	Number of focal areas per cm ² of tissue section										
	1-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	40-44	45-50	>50
Score	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0

3.3.7 Statistical Analysis

The correlation between VEGF-A levels and other prognostic variables was statistically analysed by means of Pearson's χ^2 test, Spearman rank correlation analysis and invariable analysis. Survival rates were examined using Kaplan-Meier plots of analysis. The statistical significance of differences between the survival rates of groups with different VEGF expression i.e. high or low expression was assessed by the log-rank test. The independent prognostic significance of variables was assessed by multivariate analysis, by means of a multivariate Cox regression model. P values of <0.05 were considered statistically significant. All statistical analysis was carried out using SPSS version 15.0.

3.4 RESULTS

3.4.1 Clinicopathological Characteristics

The mean age of the patients at diagnosis was 58 years (range 22-82years). Serous cystadenocarcinoma was the commonest histological subtype (55%) followed by undifferentiated (10%), endometrioid (11%), mucinous (14%), clear cell (10%). All patients were treated surgically in the first instance. Of these 70% had their tumour optimally debulked ($< 2\text{cm}$ residual disease). Clinicopathological staging showed that the majority of patients had advanced stage disease (66% and 18% for stage 3 and 4 respectively) whereas 16% had early stage disease (10% and 6% for stage 2 and 1 respectively). 70% of the tumours were poorly differentiated (grade 3), 22% were moderately differentiated grade 2, and 8% were well differentiated (grade 1). The results are summarised in Table 3.3

Table 3.2. Patient characteristics

Patient characteristic		Number n=105
Age	Mean age	58
	Range	22-82
FIGO stage	1	6 (6%)
	2	10 (10%)
	3	70 (67%)
	4	19 (17%)
Histological grade	1	8 (7%)
	2	23 (22%)
	3	74 (71%)
Level of debulking	Residual tumour >2cm	31 (30%)
	Optimal debulking <2cm	74 (70%)

3.4.2 VEGF Analysis

Patients were divided into two groups based upon the intra-tumoural VEGF expression, the median value of the VEGF score, which was 3, was taken as the cut off point. Therefore the groups were:

- 1) High VEGF expression; score of 3 or > 3.
- 2) Low VEGF expression score of <3.

No relationship was observed between intra-tumoural VEGF levels to the age of the patient ($p= 0.936$) or to the postoperative residual tumour ($p= 0.142$), the histological grade ($p= 0.15$) the histological subtype ($p=0.371$) or the FIGO stage of tumour ($p=0.18$). Association between these factors was measured with the spearman rank correlation coefficient, p values less than 0.05 were considered to be significant. The results are summarised in Table 3.4.

Table 3.3. Association of the degree of immunostaining for VEGF and clinico-pathological variables.

		Low VEGF expression (%)	High VEGF expression (%).
FIGO stage	Stage 1 (n=6)	48	52
	Stage 2 (n=10)	57	43
	Stage 3 (n=70)	39	61
	Stage 4 (n=19)	32	68
Tumour grade	Grade 1 (n=8)	47	53
	Grade 2 (n=23)	34	76
	Grade 3 (n=74)	59	41
Histological subtype	Serous (n=58)	37	63
	Endometrioid (n=12)	69	31
	Clear cell (n=11)	41	59
	Mucinous (n=8)	55	45
	Undifferentiated (n=11)	33	67
Level of debulking	<2cm (n=74)	62	38
	>2cm (n=31)	44	56

p> 0.05 for all above subgroup comparisons

Survival analysis revealed that patients that had low intra-tumoural VEGF expression had a median survival time of 36.7 months (SD 21.7) whereas the median survival of patients with a high VEGF expression was 10.8 months (SD 7.8). This difference in survival was shown to be statistically significant by Cox Proportional Hazards model after tumour stage was accounted for (p< 0.001). A Kaplan Meier survival plot is shown in Figure 3.1.

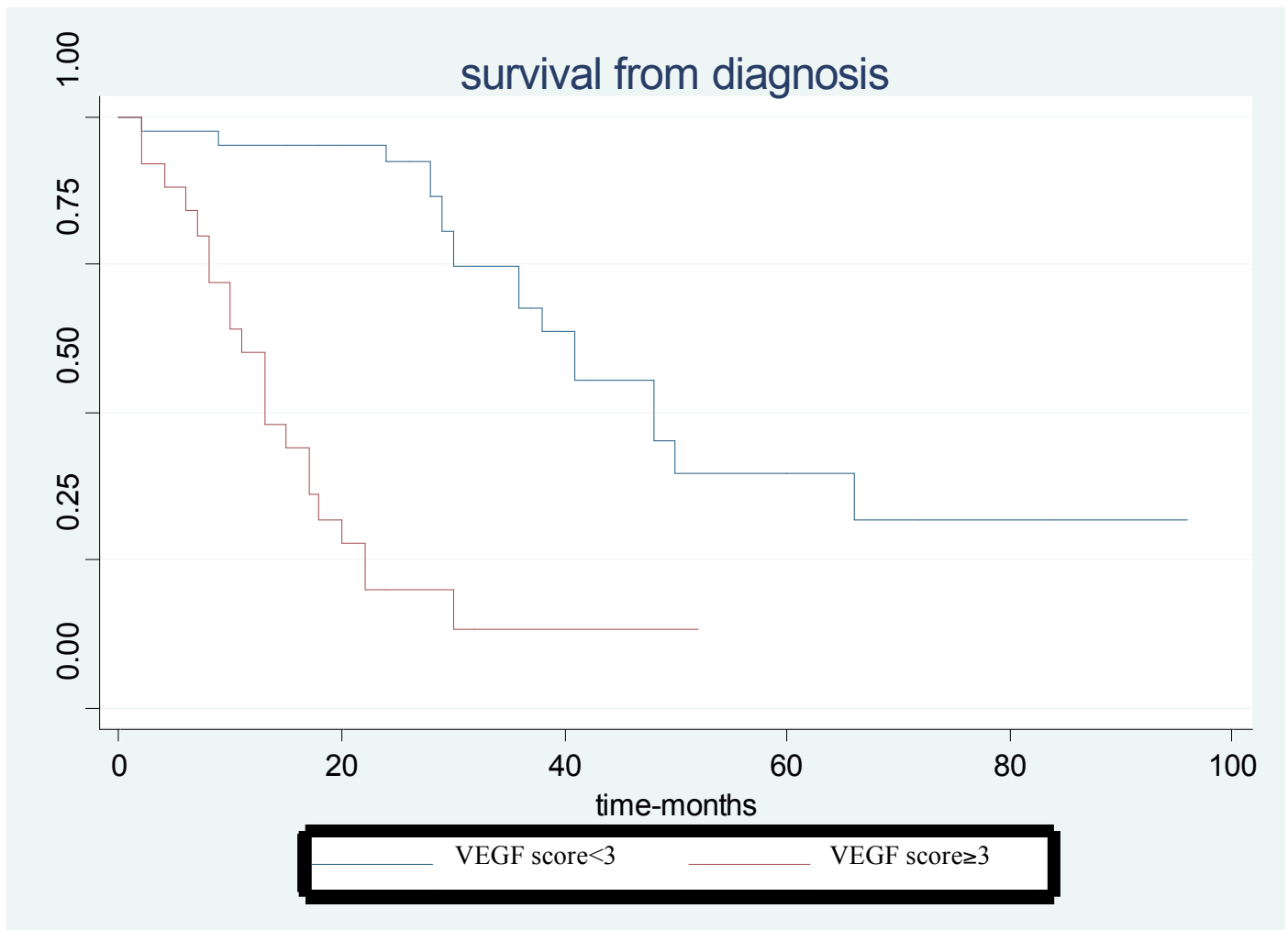


Figure 3.1: Differences in survival between high and low VEGF scoring ovarian tumours.

3.6 DISCUSSION

The central concept that tumour growth is angiogenesis dependent is widely accepted today. Every increment of tumour growth requires an increment of vascular growth^{1 97 378}.

Angiogenesis was suggested as a prognostic factor in various solid tumours but its clinicopathological significance in ovarian cancer and its exact role is

still unclear. The results of various studies are contradictory. Hollingsworth et al assessed tumour vascularity in 43 patients with advanced stage EOC, by using microvessel counts. It was reported that microvessel counts and stage were associated with disease free survival and overall survival by Kaplan Meier analysis. In this study, with Cox Proportional Hazards model, stage was the best predictor of overall survival, however the average microvessel count, which is a surrogate measure of the degree of angiogenesis, was found to be the best predictor of disease free survival.²⁸⁹ In a study by Alvarez et al overall, median survival was 2.7 years in women with cancers containing high microvessel counts versus 7.9 years in those with low microvessel counts ($P = 0.03$). A low microvessel count was associated with better 5-year survival in both early stage (I and II) and advanced stage (III and IV) disease. However in a study by Obermair et al, MVD failed to show prognostic significance in EOC, in multivariate analysis.^{291 379}

Our results indicate that patients with tumours that express high levels of VEGF have a worse overall survival rate compared to those with low VEGF or no VEGF expression. A median survival advantage of approximately 25 months is seen with the patients who have low VEGF expression ($p < 0.001$). This result was further substantiated when a multivariate Cox regression model was constructed in which established prognostic factors were accounted for. VEGF expression maintained statistical significance with regards to patient survival ($p < 0.001$). These results exhibit that immunohistochemical assessment of VEGF expression within a tumour can display further information about the potential for anti-angiogenic activity, and its effect on tumour behaviour and subsequent prognosis for the patient. The literature to date has been unclear as to the role of VEGF expression within ovarian tumours. Some studies have shown that it is a significant independent prognostic factor. Paley et al found that patients with early stage disease (FIGO stage 1 and 2) showed poorer prognosis with increased VEGF expression within the tumour³⁰¹. Shen et al showed that survival of patients with high VEGF expression was significantly worse than those with a low VEGF expression, and in multivariable analysis VEGF expression together with stage was an independent prognostic indicator for overall survival³⁸⁰.

Interestingly there was no correlation with microvessel density contradicting previous work. Raspollini et al showed that VEGF and MVD were both independent predictors of survival in advanced disease (FIGO stages 3 and 4), and also correlated with the likelihood of response to chemotherapy³⁸¹. In contrast, some authors showed no independent relationship with prognosis^{293 382}.

In our study we showed that the prognostic effects of VEGF, which are seen in all disease stages, are independent of established clinicopathological variables, such as stage, grade and residual tumour status.

From our work we have illustrated that there were no associations between VEGF and any of the clinicopathological variables, including stage and grade of the tumour. This is in agreement with other published work^{289 293 383-385}. Some studies have suggested that stage and grade are associated with VEGF expression, although these studies had a large proportion of early stage disease³⁸⁰. Heterogeneous expression of VEGF, difficulties in maintaining a standardised scoring system for the quantitative assessment of VEGF, and small sample size, may be the factors accounting for the inconsistent results among the studies thus far³⁸⁶.

Due to the central role of angiogenesis in cancer progression, inhibition of tumour angiogenesis could be a therapeutic tool to arrest tumour progression. Bevacizumab, is a monoclonal antibody against VEGF-A and is the first anti-angiogenic drug in oncology, having been approved by the FDA for that purpose. Previous studies have shown that in tumours VEGF is over-expressed in solid tumours including breast and colorectal cancer. In EOC over-expression is associated with ascites formation, malignant progression and poor prognosis. Preclinical models of solid tumours show that anti-VEGF therapy causes slowing of tumour progression, resolution of malignant effusions, and synergy with cytotoxic agents^{92 121}. Randomized trials of bevacizumab have established its efficacy in colon, breast, lung and renal cancer¹²¹. Monk et al have shown some clinical benefit from using bevacizumab in recurrent ovarian cancer³⁸⁷. There are currently ongoing trials

with bevacizumab, VEGF Trap (VEGF receptor decoy), and other agents targeting the VEGF pathway³⁸⁸. The GOG 218 and ICON 7 trials are ongoing at present and are studying the addition of bevacizumab to front line chemotherapy.

Further evaluation and research is required in prospective studies to substantiate the finding that the intra-tumoural expression of VEGF in patients is of clinical relevance, and in our work has shown to provide a survival advantage of 25 months in those patients with low VEGF expression. This can play a role in predicting a subgroup of patients who are amenable to anti-angiogenic targeted therapy and may help to stratify patients into those who would benefit from first line anti-angiogenic therapy, in addition to standard chemotherapy.

In conclusion the expression of VEGF-A is an independent prognostic indicator in our series of patients, with all stages of ovarian cancer. The angiogenic evaluation of the tumour, by means of VEGF expression may help to identify a group of patients in which anti-angiogenic therapy is more effective, and could be used as a first line agent, either alone or in combination with standard platinum based chemotherapy. This observation in turn leads to the question that whether the assessment of VEGF-A, is something that should be performed prior to introducing chemotherapy?

Chapter 4: ANGIOGENESIS AND RESPONSE OF OVARIAN CANCER TO PLATINUM BASED CHEMOTHERAPY

Publication:

Immunohistochemical Expression of VEGF Predicts Response to Platinum Based Chemotherapy in Patients with Epithelial Ovarian Cancer. G.K Siddiqui, A.B Maclean, K.Elmasry, A.Wong te Fong, R.W Morris, M. Rashid, R.H.J. Begent, G. M. Boxer. Angiogenesis 2011 May;14(2):155-61.

Oral Presentations

VEGF Expression as a Biomarker to predict disease response to Platinum based Chemotherapy in Epithelial Ovarian Cancer. Siddiqui GK, Wong Te Fong, Morris R Maclean A.B. Royal College of Obstetricians and Gynaecologists Scientific Meeting, Montreal, Canada, September 2008.

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4.1 INTRODUCTION

Epithelial Ovarian cancer EOC is the leading cause of death among the female genital tract malignancies ³⁸⁹. The high mortality of ovarian cancer is due partly to the fact that most patients are detected when the disease is at an advanced stage (III and IV).

Developments in chemotherapy and surgical technique have had minimal effect on patient survival over the last few decades ³⁸⁹. Cytoreduction together with a combination of taxane and carboplatin is the standard treatment for EOC. However approximately 50% of patients with advanced disease will relapse despite this form of management. Moreover 15-20% is resistant to platinum based treatment ³⁹⁰.

Tumour stage and residual tumour mass following primary cytoreductive surgery has shown to reliably predict outcomes in patients with EOC, however this offers no information with regard to the potential sensitivity to molecular targeted therapies or indeed standard platinum based chemotherapy. Investigation of novel prognostic markers offers an insight into the mechanisms of tumour development and suggests potential avenues for the development of new therapeutic agents particularly through the use of monoclonal antibody therapies targeted against antigens that are overexpressed in disseminated cancer cells ³⁹¹.

Angiogenesis has a vital role in tumour growth, invasion and metastases ³⁹²⁻³⁹⁴. This process is tightly regulated in normal tissues for maintenance and repair, and in wound healing, by the VEGF signalling pathway. VEGF, originally identified as a vascular permeability factor ³⁸³, is a homodimeric glycoprotein that is expressed in a wide variety of normal and transformed cell types ³⁹⁵.

The VEGF signaling pathway plays a crucial role in angiogenesis in both normal and disease settings, triggering multiple signaling pathways that can

profoundly affect the function of endothelial cells leading to cell survival ³⁹⁶, migration ³⁹⁷, mitogenesis ³⁹⁸ and differentiation ³⁹⁹. The VEGF-related gene family of angiogenic and lymphangiogenic factors (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor - PlGF) are secreted as glycoproteins and mediate their effects by selectively interacting with one of the three known VEGF tyrosine kinase receptors (VEGFR). VEGF-A (made up of 5 isoforms of varying molecular weight – kDa 121; 145; 165; 189; 206:), VEGF-B (made up of 2 isoforms of 167 kDa and 186 kDa) and PlGF, all bind to VEGFR-1. VEGF-A, VEGF-C; VEGF-D and VEGF-E bind to VEGFR-2, and VEGF-C and VEGF-D to VEGFR-3 ³⁴⁰.

Angiogenesis is vital for the growth and progression of tumours for their development from a small-localised lesion to a tumour with the ability to metastasise ⁴⁰⁰, and its expression has been shown to occur in response to up regulation of hypoxia-inducing factor (HIF-1 α)³⁸². VEGF expression has been shown to predict for metastases and has prognostic significance in various solid tumours. Several clinicopathological studies have shown a direct association between expression of VEGF and it's receptors (VEGFR1 - 3) and microvascular density (MVD), demonstrating the importance of studying intratumoural angiogenesis ^{286 401}.

Thus, demonstration of over-expression of VEGF in patients' tumours by immunohistochemistry (IHC) not only provides the basis for a biomarker assay for assessing disease progression, but also identifies rationally those patients who might benefit from anti-angiogenic therapy.

4.1.1 Chemotherapy for epithelial ovarian cancer

The majority of patients with EOC present with advanced stage disease, and although response rates are high, many patients eventually develop drug-resistant tumours and the 5-year mortality remains at 60-70%.

Platinum drugs are the most active agents in EOC and form the mainstay of any regimen for this disease. Cisplatin and carboplatin have dominated the drug therapy of ovarian cancer during the past three decades. In 1978, a conference sponsored by the Division of Cancer treatment, National Cancer Institute (NCI), clinically evaluated the heavy metal derivative cis-diamminedichloroplatinum (DDP). Following thorough laboratory studies the National Cancer institute introduced it clinical practice in 1971 ⁴⁰².

Cisplatin was discovered in the late 1960's by Barnett Rosenberg, who went onto to interest the NCI to test DDP in it preclinical mouse models and to foster its development ⁴⁰³. In 1979 FDA approval was obtained for the treatment of ovarian, testicular and head and neck cancers.

4.1.1.1 Mechanism of Action

Three platinum compounds are in common use, and include cisplatin, carboplatin and oxaliplatin. It is generally accepted that DNA is the most important intracellular target of platinum based compounds. However DNA is not the only target; binding to protein and RNA also occurs, as has been shown by various studies ^{404 405}. In addition it can also not be excluded that damage to cell membranes is also relevant. Even though there are various studies supporting the notion that binding of platinum compounds to DNA leads to the killing of the tumour cell and the observed side effects seen in patients treated with cisplatin, this does not exclude other modes of action to achieve cytotoxicity ⁴⁰⁶⁻⁴⁰⁹.

It is evident that on route to the tumour cells, the platinum compounds can interact with blood products and with cell wall components. These reactions might also contribute to the toxic side effects observed during the treatment of patients with platinum compounds. Apart from binding of platinum compounds to DNA in the tumour cell-which is generally believed to be the origin of anti-

neoplastic activity-several other events will take place after the administration of the drug into the body. The circulating platinum species is transported-most likely as a neutral species-into both tumour cells and normal cells, and then undergoes hydrolysis inside the cells. Subsequently the hydrolysed platinum species can react with all kinds of cell components, such as peptides, proteins, DNA and RNA. Although the binding of the DNA appears to be responsible for the ultimate cell killing, it cannot be excluded that reactions of the platinum species with other molecules are leading to toxic side effects. The repair of DNA may lead to platinum resistance. Also the transport of platinum into the cell has been increasingly investigated. It has been shown that 25% of proteins in the cell are concerned with transport. Considerable advances have been made recently in defining the role of transport into the cell and export or sequestration of platinum as another determinant of drug sensitivity and resistance; it has become clear that platinum utilizes the copper transporters and exporters as well as other cation transporters⁴¹⁰. Manipulations in transport can alter cell sensitivity.

4.1.1.2 Cisplatin and Carboplatin

Cisplatin (*cis*-dichlorodiammine platinum(II)) was discovered serendipitously by Rosenberg in the 1950's while investigating inhibition of bacterial growth by electric currents²⁶³. The resulting inhibitory complexes were produced by the reaction of platinum electrodes with growth media. Cisplatin, a neutral complex, was the most active of these, and was tested in clinical trials²⁶⁴.

It is thought that cisplatin enters the cell by both passive diffusion and facilitated transport, possibly by a gated channel⁴¹¹. Some of the uptake is dependent on energy because pharmacological agents such as ouabain (a Na⁺, K⁺-ATPase inhibitor) reduce it⁴¹².

Cisplatin is a water-soluble complex, which has a central platinum atom surrounded by two chlorine atoms and two ammonia groups²⁵³ (Figure 4.1).

When cisplatin enters the cell, the chloride ions dissociate (due to the low concentration of intracellular chloride ions) leaving a reactive diamine-platinum complex, which reacts with water and then, can interact with DNA²⁵³. Cisplatin binds preferentially to the N7 atom of guanine and adenine residues⁴¹³, although there are numerous potential reaction sites in all four bases⁴¹⁴.

Cisplatin is widely used not only for the treatment of ovarian cancer, but also for many other solid tumours such as those arising from the testes, bladder, lung, and head and neck. Cisplatin was first introduced in 1973 for treatment of ovarian cancer, which improved disease-free survival and response rates when compared to the then-standard treatments²⁵². Cisplatin was either used alone or in combination with cyclophosphamide. Cisplatin causes very severe nausea and vomiting, and is highly nephrotoxic²⁵³.

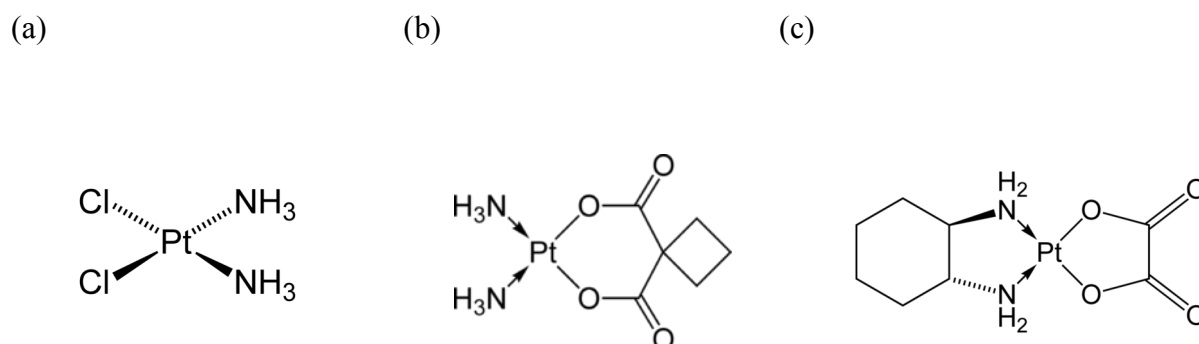


Figure 4.1: Structure of (a) cisplatin (b) carboplatin (c) oxaliplatin

In the early 1980's carboplatin, which is a derivative of cisplatin, was introduced (Figure 4.1). Carboplatin causes less nephrotoxicity, neurotoxicity, and ototoxicity, and less severe nausea and vomiting than cisplatin, but it is more myelotoxic²⁵³. Tinnitus and hearing loss in the high frequency ranges may occur, as may peripheral neuropathies, hyperuricaemia, myelotoxicity and nephrotoxicity.

The cytotoxicity of cisplatin and carboplatin are believed to result from the formation of platinum-DNA adducts. These include monoadducts, intrastrand crosslinks, interstrand crosslinks (ICLs), and DNA-protein crosslinks⁴¹⁵ (Figure 4.2). There has been much debate about which adduct is responsible for the cytotoxicity of cisplatin. Fischtinger-Schepman *et al* concluded that 1,2 intrastrand crosslinks produced by cisplatin were responsible for its cytotoxicity because the peak of formation of these lesions co-incided with the peak of cytotoxicity in Chinese hamster ovary cells (CHO)⁴¹⁵. Zamble *et al.*, demonstrated that the 1,2-cisplatin adducts are poorly recognised, adding support to the argument that they are a critical cytotoxic lesion⁴¹⁶. In contrast Zwelling L.A *et al* found a correlation between cytotoxicity and ICLs in L1210 cells *in vitro* using alkaline elution⁴¹⁷. Meyn *et al* demonstrated that an ERCC1 mutant CHO cell line, UV20, was extremely sensitive to cisplatin and defective in the uncoupling of ICLs, which was taken to demonstrate a direct relationship between sensitivity and ICL repair⁴¹⁸. In the nitrogen mustard class, there is a clear correlation between the extent of interstrand crosslinks (ICL) and cytotoxicity⁴¹⁹. However, the platinum drugs produce a high proportion of intrastrand crosslinks that clearly contribute to their activity⁴²⁰.

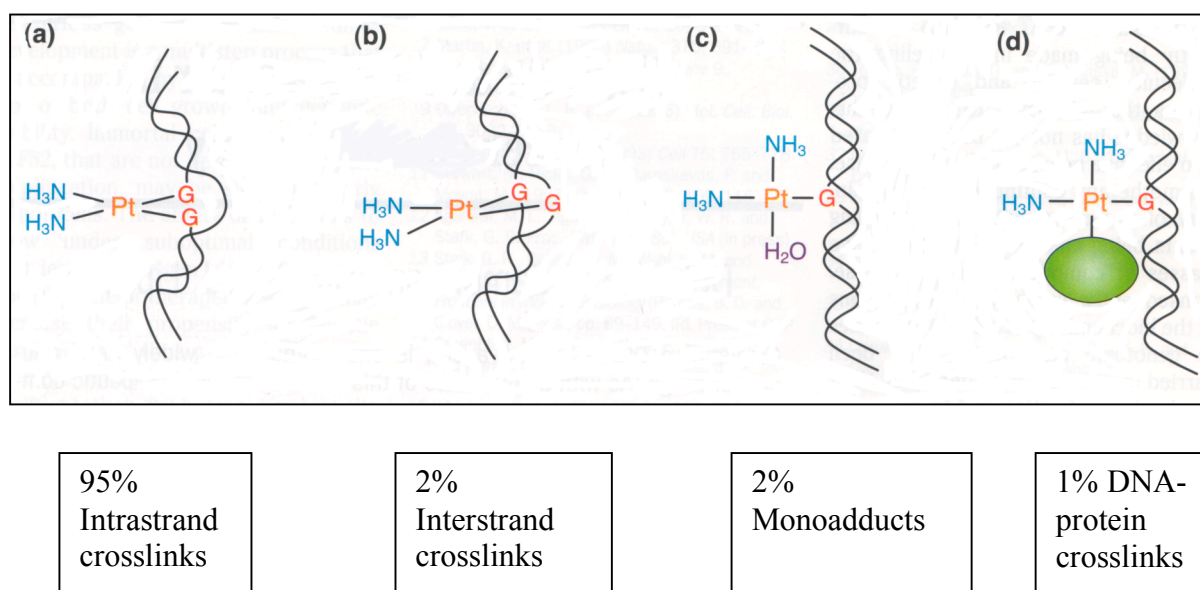


Figure 4.2: The different types of DNA adducts produced by cisplatin, and frequencies of occurrence.

Carboplatin has a much slower rate of aquation and ICL formation compared to cisplatin⁴²¹, and therefore the time to reach the peak of ICL formation is much longer with carboplatin.

4.1.1.3 Oxaliplatin

Oxaliplatin is an analogue of cisplatin that was developed to try and overcome drug resistance (Figure 4.1). It has a different spectrum of anti-tumour activity and has shown activity in treatment of advanced colorectal cancer either as a single agent or in combination with 5-fluorouracil⁴²². Side effects of Oxaliplatin include neurotoxicity, including facial dyesthesia, which may be provoked by cold weather, and peripheral sensory neuropathy⁴¹².

4.1.2 INDICATIONS FOR CHEMOTHERAPY

4.1.2.1 Early Disease

The use of chemotherapy in the treatment of advanced epithelial ovarian cancer is established (FIGO stages III and IV). The role of therapy in early stage disease remains uncertain. It would be useful to predict out a subset of patients with early stage disease that are at the highest risk of relapse, in order to offer them adjuvant chemotherapy. Patients who have stage IA and IB disease are generally managed without chemotherapy, unless there are adverse prognostic features. Patients with stage IC disease are given adjuvant chemotherapy, as it has been shown that presence of peritoneal fluid with cancer cells (Stage IC) reduces survival²³⁷. It has been suggested that stage I patients with well-differentiated tumours have a 90% 5-year survival rate, and currently chemotherapy is not thought to be of benefit in these women.

4.1.2.2 Advanced Disease

The mainstay of treatment of patients with advanced disease (Stage II and above) is to undertake optimal cytoreductive surgery followed by platinum based chemotherapy. Cytoreductive surgery and volume of residual disease post procedure has prognostic significance. If chemotherapy is given, those with no macroscopic disease postoperatively have a progression free survival of 42 months versus 20 months for those with disease of 1cm diameter or less. These had an improved survival over those with disease greater than 1cm in diameter. A majority of patients with stage III disease will still die from their disease although combination chemotherapy has improved their survival with 25-40% being alive at 5 years. Stage IV disease has a poor outcome, with a median survival of 12 months and only 5-10% alive at 5 years ⁴²³. When cisplatin was introduced into first line trials in ovarian cancer, there was little difficulty in recognizing its impact in progression free survival (PFS). Although its impact became increasingly clear, its initial use was initially entangled in an array of complex combinations. Studies by the GOG employed it in combination with cyclophosphamide and this doublet became the reference regimen for subsequent trials until GOG 111 showed the PFS and overall survival OS superiority of cisplatin and Paclitaxel ⁴²⁴. Eventually a toxicity reduction but similar disease response showed carboplatin and paclitaxel was preferred – because of reduced side effects and toxicity with at least equal efficacy over cisplatin ²²³.

The role of other cytotoxic drugs in current use for ovarian cancer in complementing the effects of platinum drugs is less clear. After exposure to cisplatin, various alkylating agents had disappointing activity, and doxorubicin's acute and chronic toxicities led to reduced use until the advent of liposomal formulations. After the introduction of paclitaxel, this was followed by Topotecan, gemcitabine, and pegylated liposomal doxorubicin (PLD) drugs with reproducible activity and reasonable therapeutic index came into common use. However the addition of the latter three in the five-arm GOG 182 did not prove advantageous over the carboplatin and paclitaxel doublet. When used as short term consolidation or maintenance after initial platinum-

based induction, only paclitaxel has shown improvement in PFS (in GOG 178 that was stopped early).

4.1.3 Drug resistance in ovarian cancer

Chemotherapy resistance is clinically defined as the progression of disease during therapy, absence of regression during therapy, or recurrence within 6 months after completed treatment. Progression of disease during therapy is defined by the RECIST (response evaluation criteria in solid tumours) guidelines as a 20% increase in the sum of the longest diameter of the target (i.e. ovary) or non-target lesions⁴²⁵. Absence of regression during therapy is defined by the RECIST guidelines as neither disappearance of all target lesions (complete response), >30% decrease in the sum of the longest diameter of target lesions (partial response), or progressive disease (defined above)⁴²⁵. Recurrence of disease is confirmed as a rise of serum Ca-125 levels to more than twice the upper limit of normal²⁴⁴, and in patients with persistently elevated Ca 125 serum levels, recurrence of disease is a doubling of Ca 125 above the nadir⁴²⁶. Recurrence of disease can also be confirmed by CT scans.

Tumours are considered to be sensitive if they exhibit complete clinical response to therapy, or if relapse occurs after remission and treatment has not been administered for more than 6 months⁴²⁷. Resistance to cisplatin can be intrinsic or acquired. Intrinsic is present at the time of diagnosis, and patients fail to respond to first line chemotherapy. Laboratory studies have shown that cisplatin resistance is multifactorial, consisting of mechanisms such as: (1) decreased drug accumulation (2) increased drug inactivation (3) evasion of apoptosis (4) enhanced ability to repair DNA damage. However, because cancer cells are heterogeneous, more than one mechanism of drug resistance may be present at one time.

4.2 AIM

This study was undertaken to examine whether there is an association between VEGF-A expression in epithelial ovarian cancer and the tumour's response to platinum based chemotherapy.

4.3 MATERIALS AND METHODS

4.3.1 Study Population

The study cohort consisted of 66 cases with advanced Epithelial Ovarian Cancer (EOC) (FIGO stages III and IV), who underwent surgical staging and tumour debulking between for primary EOC at the royal Free NHS Trust between 1995 and 2000. The inclusion criteria for the cases included:

- a) FIGO stage III / IV disease.
- b) Underwent cytoreductive surgery
- c) Completed 6 cycles of adjuvant platinum based chemotherapy.
- d) Did not receive neoadjuvant chemotherapy.

Clinical data and patient information were collected from the patient's clinical records. Ethical approval was obtained for this retrospective analysis from the Royal Free Hospital and Medical School Research Ethics Committee (code number 09/H0720/87). Informed consent was obtained as per local guidelines.

4.3.2 Chemotherapy Treatment

All patients in this study received platinum based primary therapy with carboplatin. In the majority of cases this was in combination with taxanes

(56/66 patients, 83%). 10 patients received carboplatin alone. All patients received 6 cycles of primary therapy. 30 patients were given further consolidation therapy after completing primary therapy. Because consolidation therapy was not given as standard treatment to all patients, completion of primary therapy was used as the end point for determining response outcomes.

4.3.3 Histology and Immunohistochemistry

The paraffin blocks from each case were reviewed by a pathologist (JCC) and representative areas of tumour were chosen after examining the haematoxylin & eosin stained slides, by two observers, including one pathologist (JCC and AC). Briefly, sections of primary tumour from each case were cut at 3µm and were deparaffinised in xylene, rehydrated with graded ethanol, and immersed in 0.3% H₂O₂ in methanol for 20 minutes to inhibit endogenous peroxidase activity. Sections were then incubated with commercially available mouse monoclonal antibody to VEGF-A (R&D Systems Abingdon, UK.) for 45 minutes at RT and binding was then visualised by sequential incubation with a biotinylated labelled secondary antibody followed by streptavidin-biotin peroxidase labelled complexes reagent (Dako Ltd, UK). IHC reactivity was then visualised using 0.1% Diaminobenzidine tetrahydrochloride in 0.03% HCl. Negative controls were prepared by omitting the primary antibody (to VEGF) from the procedure and substituting with mouse immunoglobulin (class IgG2b).

4.3.4 VEGF Quantification

For the assessment of VEGF, regions of interest (ROIs) containing tumour cells with minimal or no associated connective tissue stroma were defined by two independent observers (JCC & GKS). Each slide that contained tumour was analysed by using an eyepiece graticule that defined an area of 1cm x

1cm at a magnification of 200 times (with x20 objective). The graticule comprising 100 individual squares (each 1mm x 1mm) was placed over three representative fields of the slide that contained tumour (minus large areas of connective tissue). The identification of at least 1 VEGF-positive cell within the confines of an individual square defined that square as a positive focal area. The number of VEGF-positive focal areas was totalled for each slide and then an average number of focal areas for that case was obtained. Using the tariff shown in Table 4.1 (scoring system devised by GKS and JCC), the average number of focal areas per case was converted into a score for VEGF, on an increasing linear scale of 0 – 5, (where 0 = 1 - 4 focal areas, and 5 =>50 focal areas). All observations and analysis was performed by two independent observers (GKS and JCC) who were blinded to all clinical data. In all cases there was less than 5% inter-observer variation in results.

Table 4.1. VEGF score by IHC as a function of the number of focally positive VEGF areas

	Number of focal areas per cm ² of tissue section										
	1-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	40-44	44-50	>50
Score	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0

4.3.5 Statistical analyses

Statistical analyses were performed using SPSS version 16.0. Expression of the intratumoural VEGF-A protein was correlated with platinum sensitivity (using a Mann-Whitney U Test and Fishers Exact Test). Spearman Rank correlations were calculated to test for association between VEGF-A expression and, patient age, tumour stage, grade and survival. Survival times were estimated in months from the date of operation to the date of death (or date of last follow-up). Kaplan Meier survival plots and a log rank test were

used for the univariate analysis of VEGF expression in resistant and sensitive groups. The Cox Proportional Hazard Model was used for multivariate analysis. Response to therapy was defined as for the 2003 revision of the 'Gynecologic Oncology Group' criteria³⁶². Patients who had progressed on chemotherapy or who relapsed within 6 months of completing of completing their primary therapy were defined as platinum resistant. All other patients were categorised as responders to platinum-based therapy. Recurrence of tumour was defined by imaging modalities (CT & Ultrasound) and/or by the demonstration of increasing levels of CA-125 in the serum.

A cut-off point for scoring of VEGF as, high expression (>3) and low expression (≤ 3) was used to statistically analyse the data. A p (probability) value of <0.05 was considered to be significant for all statistical tests.

4.6 RESULTS

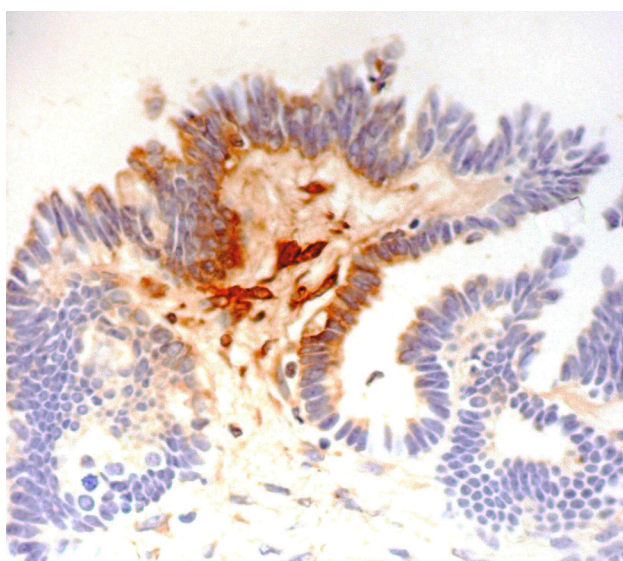
All patient characteristics are detailed in Table 4.2. The median age of the patients was found to be 53 years (range 22 -82 years). Of all the EOC patients in this cohort, 66% were defined by pathology as serous, 15% as endometrioid, 13% as mucinous, and 6% as clear cell. Seventy-five percent of the patients were optimally debulked at primary surgery with less than 2cm^3 residual disease, whilst 25% had greater than 2cm^3 residual disease after debulking. Sixty-four percent of the patients were defined pathologically as having grade 3 histology. Using Gynecologic Oncology Group (GOG) criteria the patients were stratified into two groups based on their response to platinum-based chemotherapy (see Materials and Methods). 45 patients were found to have platinum-sensitive disease (68%), the remaining 21 patients being platinum-resistant (32%).

Table 4.2: Patient Demographics.

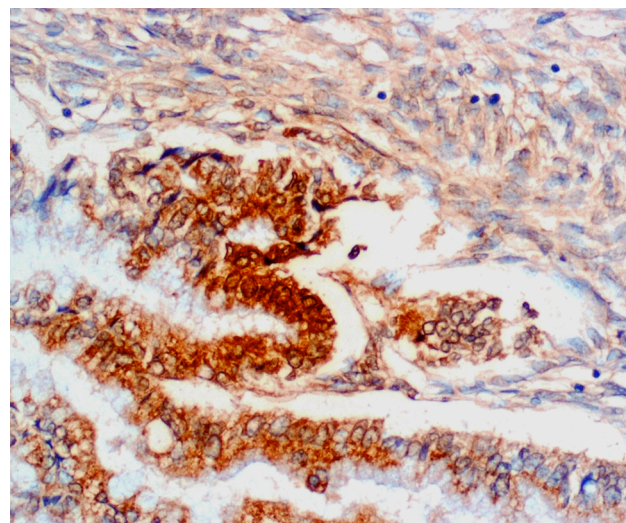
Characteristic		Patients n=66 (%)
Age	Median	53 years
	Range	22-82 years
Histological subtype	Serous	44
	Endometrioid	8
	Mucinous	9
	Clear cell	4
Extent of residual tumour	>2cm	25%
	<2cm	75%
FIGO stage	3	80%
	4	20%
Clinical response to platinum chemotherapy	Sensitive	45 (68%)
	Resistant	21 (32%)

4.6.1 VEGF expression and Primary response to Chemotherapy

Immunohistochemical reactivity of VEGF in the tumour cells was granular in nature and mainly confined to the cytoplasm of cells (Figure 1a and b). Binding of antibody to VEGF was observed in a few areas of the connective tissue stroma in some cases, and many cases demonstrated heterogeneous reactivity for VEGF with only focal areas of the tumour showing positivity (Figure 1a). The median VEGF score in the platinum chemoresistant and platinum chemosensitive groups was 4 and 2, respectively. The range for the number of focal positive areas of VEGF reactivity for the platinum-resistant group was 22 - 150, compared to 5 - 34 for the platinum-sensitive one.



(a)



(b)

Figure 4.3: Microscopy images of sections reacted by immunohistochemistry for VEGF in cases of epithelial ovarian cancer demonstrating (a) heterogeneous expression of VEGF in a case from the group with low levels of reactivity (VEGF score ≤ 3) and (b) homogeneous expression of VEGF in a case from the group with high levels of reactivity (VEGF score > 3). Magnification x 200. VEGF reactivity is brown. Nuclei of cells (haematoxylin-stained) is blue.

4.6.2 Statistical Analysis

The VEGF levels were significantly lower in the chemosensitive cases compared to those recorded in the chemoresistant patient group (Mann Whitney U test, $p = < 0.0001$). The VEGF score was also tested in a multivariate regression model and demonstrated that VEGF expression within the tumour was a significant factor for predicting response to platinum-based chemotherapy. No relationship was observed between VEGF expression and the age of the patient, or the stage or grade of the tumour and they were not significant predictors of disease response to chemotherapy, in both univariate and multivariate analysis.

4.6.3 VEGF-A expression and overall survival

To examine the relationship between the level of VEGF in the tumour and overall survival, patients were stratified into two groups based on the extent of VEGF expression. Patient survival was recorded as months of survival (with or without disease after surgery), or months from surgery to death. Patients were further monitored with a median follow-up period of 18 months (range 12 - 33 months). Overall median survival was 10 months in patients with a high tumour VEGF score in the tumour (i.e. >3) versus 36 months in patients with a low tumour VEGF score (≤ 3).

VEGF levels (IHC score) was significantly inversely correlated with survival in months, in all patients (using Spearman Rank Correlation Test, $p = < 0.01$), and also higher levels of VEGF were associated with the poor outcome group (i.e. died of their disease), $p = < 0.01$ (Mann Whitney U test).

Overall, of the 66 patients, 39 patients died of their disease within the length of time of the study. Response to platinum treatment (as assessed by CT and ultrasound and/or by demonstration of increasing levels of CA125 in serum) was significantly correlated with survival (Cox Proportional Hazards model, $p = < 0.0001$). VEGF expression (IHC score) was also found to be significantly correlated to overall survival ($p < 0.0001$) after tumour stage was taken into account (Cox Proportional Hazards model, $p = < 0.0001$). Kaplan-Meier plots of survival with reference to platinum sensitivity and VEGF status is shown in Figure 4.4.

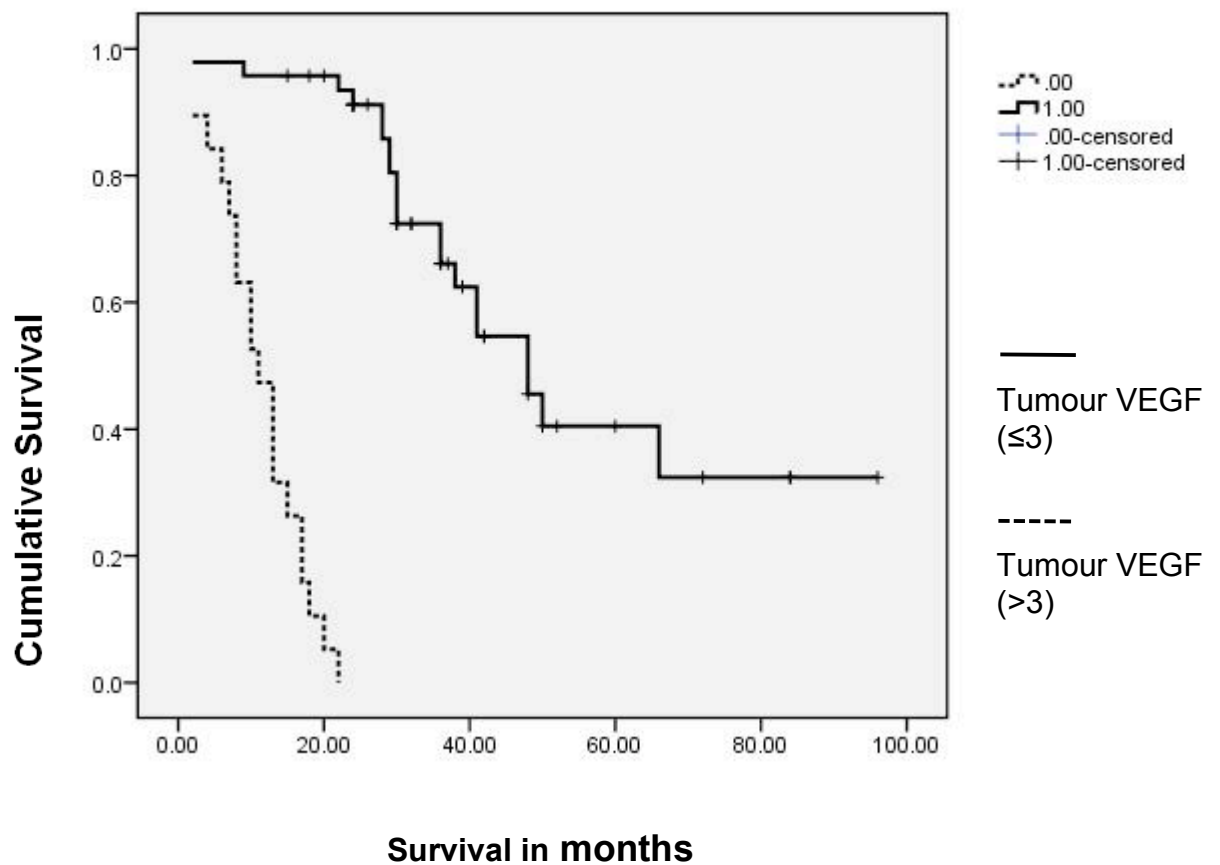


Figure 4.5. Kaplan-Meier plots showing the relationship of VEGF status (by IHC) with survival, in 66 patients with epithelial ovarian cancer.

4.7 DISCUSSION

EOC is mainly treated by platinum-based chemotherapy and this study has evaluated the expression of VEGF as a marker of chemotherapeutic responsiveness in such patients. These data strongly suggest that in EOC immunohistochemical level of VEGF expression is significantly associated with platinum sensitivity and overall patient survival with high tumour VEGF

expression predicting for primary resistance to platinum-based chemotherapy. This study has also demonstrated by multivariate analysis that VEGF levels is an independent predictive factor for response to platinum-based chemotherapy. Overall, 86% of patients who were resistant to platinum were found to have a high VEGF score, compared to only 2% in the platinum-sensitive group ($p < 0.0001$). We have also demonstrated that VEGF expression within the tumour significantly correlated with overall patient survival ($p = < 0.0001$). Overall median survival was 10 months in patients with a high VEGF score in the tumour versus 36 months in patients with a low VEGF score, demonstrating a median survival advantage for the latter group of approximately 26 months. Illustration of the Kaplan-Maier plot (Figure 4) illustrates the potential power of using VEGF scoring by IHC as a prognostic biomarker for treatment response. We have demonstrated that VEGF expression is a surrogate for survival and have shown its ability to predict outcome in our cohort of EOC patients. Whilst we understand that within all IHC scoring systems, subjectivity may be a problem, and there is an element of subjective bias. We were careful to design a robust protocol that took account of this by scoring all VEGF positive areas, within all sections of tumour per case.

Angiogenesis plays a central role in the clinical behaviour of EOC and progression of disease, with VEGF-A being an important mediator in promoting and increasing vascularity. VEGF has been shown to be expressed in the majority of tumour specimens from patients with EOC, as assessed by *in situ* hybridisation³⁸² or immunohistochemistry²⁸⁶. In addition to stimulating angiogenesis, VEGF-A increases vascular permeability and is thought to be partly responsible for the development of ascites in both animal models and patients with EOC⁴⁰¹. Several studies have suggested that high levels of VEGF-A in either tumour or patient serum correlate with poor survival in this disease^{289 291 301 311}. These observations suggest that VEGF-A could be an important therapeutic target in EOC. Our study illustrated that patients with high levels of VEGF expression in the tumour have lower median survival rates compared to those patients that have low expression of VEGF. It is noteworthy that single agent bevacizumab (Avastin®), a monoclonal antibody

against VEGF-A, is active in EOC as well as in renal cancer ⁴²⁸, whereas it appears to have relatively low activity when used by it in other tumour types such as colorectal or breast cancer ^{429 430}. It is unclear what the mechanism is for this phenomenon but it may reflect a greater contribution of VEGF-dependent angiogenesis to the pathogenesis of relapsed disease and it may be related to the way that colorectal cancer and breast cancer metastasise versus the peritoneal invasion seen in ovarian cancer ²⁷⁵.

To date there have been no biomarkers established that can predict response to platinum-based chemotherapy in EOC. Currently, three biomarker assays of angiogenesis are under development, including quantification of urinary matrixmetallinoproteases ⁹³, analysis of the platelet angiogenic proteome ⁹⁴ and measurement of blood levels of circulating endothelial cell precursors (CEPs) and mature circulating endothelial cells (CECs) ⁴³¹. These methods can detect human tumours in mice at sizes of millimetres or less. In future it may be possible to detect recurrence of cancer by these and other biomarkers, to predict groups of patients likely to be resistant to platinum based chemotherapy, and to guide antiangiogenic therapy in patients with occult tumour, before clinical symptoms appear ⁸⁴.

Others have reported that the elevated expression of VEGF in the tumour of EOC patients is associated with poor prognosis ^{39 275 301 304}. Whilst VEGF has been shown to be a prognostic factor in EOC, this study adds to the field by correlating VEGF status specifically with platinum treatment response. One possible mechanism to explain the prognostic capacity of high tumour VEGF expression may be related to its function as a regulator of angiogenesis. However, the pathway that might be involved in causing platinum resistance, in association with high VEGF levels is at present unknown. In this study, it is not surprising perhaps, that poor prognosis (defined by survival) is also reflected in the patients' VEGF status (which others have also shown to be independently related to poor outcome). Whether or not VEGF overexpression and presumably stimulation of angiogenesis, can be shown to directly to modulate platinum sensitivity, *in vivo* is unclear. But, a recent *in vitro* study by Roberts et al demonstrated that clonogenic survival of colorectal

cancer cells after exposure to oxaliplatin, was significantly reduced in spheroids grown in hypoxic conditions ⁴³². Moreover, they reported higher levels of platinum adducts in aerobic cells treated with the drug.

This study is one of the first we believe, to define pre-treatment intratumoural expression of VEGF as a predictor of response to platinum-based chemotherapy.

Other recent experimental data also supports an association between VEGF and platinum response. It has been reported that an inhibitor of VEGF (soluble decoy receptor – VEGF trap) acts synergistically with Paclitaxel in a human ovarian cancer model leading to improved survival ⁵⁰. Therefore, it could be inferred that high VEGF levels in the tumour may hamper the cytotoxic effect of Paclitaxel (in the peritoneal cavity) and is one potential example of how VEGF overexpression may have implications for treatment with platinum-based agents.

Our findings suggest that the immunohistochemical assessment of VEGF within a tumour section, offers valuable information about progression of disease in EOC, and can be a valuable prognostic indicator for both the patient and clinician. Data from other groups support these findings including, Bamias *et al* who showed that high VEGF levels in the ascites of patients with advanced epithelial ovarian cancer were of prognostic significance. That group also reported an association of VEGF expression, within the ascites of patients, with platinum resistance ⁴³³.

Recent interest has focussed on the use of antiangiogenic drugs in an attempt to inhibit the pro-tumour effects of VEGF. Evidence that these targeted therapeutics were biologically active came from studies in 2004, using bevacizumab (Avastin®) - Genentech Inc. San Francisco, US) and was the first agent to target angiogenesis. It was licensed as a recombinant humanised monoclonal antibody (administered three-weekly) for the treatment of advanced solid cancers, and its mode of action is by targeting the isoforms of VEGF, which circulate in the bloodstream, preventing the ligand binding to

its cognate receptor. Avastin® has been shown to improve survival in patients with advanced disease alone, and in combination with existing chemotherapy. More recently, smaller molecular weight tyrosine kinase inhibitors (TKIs) have also shown the ability to interfere within the VEGF signaling network. This class of agents (aminophthalazines) includes Vatalinib (PTK787/ZK222584, Novartis, Basel, Switzerland) which acts by targeting VEGFR-1, -2 and -3, PDGFR- β and c-Kit, and has shown to improve progression-free survival of colorectal cancer patients when administered in combination with FOLFOX in Phase III trials ⁴²⁹. Anti-VEGF therapy causes a slowing of tumour progression, resolution of malignant effusions and synergy with cytotoxic agents ^{92 381}. It is also being studied as a first line treatment in a US trial (GOG 218) and a European Trial (ICON 7) in EOC. Currently, there are many ongoing efforts to develop biological markers that predict sensitivity/resistance to particular strategies in EOC. This study shows that measurement of the level of VEGF, determined by IHC in archival material, may be used for selection of patients who may benefit from the addition of anti-VEGF therapies to front line platinum/ paclitaxel chemotherapy.

Chapter 5: POPULATION BASED CASE CONTROL STUDY OF A VEGF GENE POLYMORPHISM AND OVARIAN CANCER RISK AMONG PAKISTANI WOMEN

Oral presentations:

- 1) 'Ovarian cancer in Pakistan: a different disease entity?' Presented at the International Gynecological Cancer Society meeting, Bangkok, 2008.
- 2) 'Population based case control study of VEGF gene dimorphism and ovarian cancer risk among Pakistani women.' Presented at the International Gynecological Cancer Society meeting, Prague, 2010.

5.1 INTRODUCTION

5.1.1 Ovarian Cancer In Pakistan

Primary carcinoma of the ovary is the fourth most common female cancer in the UK and the most common cause of death from a gynaecological malignancy. The incidence of the disease varies widely across different geographical regions and ethnic groups with the highest reported incidence rates in Europe and North America and the lowest rates in Asia ⁴³⁴. Pakistan, however, is the exception to these figures from Asia; the age standardised rate (ASR) for ovarian cancer is reported to be 10.2 per 100, 000 which is comparable to rates seen in the West ⁴³⁵. In Pakistan ovarian cancer is the most common cancer of gynaecological origin ⁴³⁶. This is in direct contrast to the developing world where cervical cancer remains the commonest gynaecological malignancy ^{434 436}.

Despite the extensive published research literature on the subject, the natural history of ovarian cancer development, including the nature of the precursor cell type, remains poorly understood. Various theories have been suggested for how ovarian cancer develops. These include the incessant ovulation theory ¹²⁴ which proposes that the risk of ovarian cancer increases as a result of the recurrent minor trauma to the ovarian surface epithelium that occurs during ovulation. The suggestion is that the greater the number of times the ovarian surface epithelium undergoes remodelling, the greater the chance will be that aberrations leading to a malignant transformation will occur.

Whilst the exact aetiology of ovarian cancer remains relatively unknown, epidemiological studies have shown that several hormonal and reproductive risk factors have been associated with the disease ^{437 438}. Based on several studies, there is good evidence that increased parity ^{439 440}, the use of the oral contraceptive pill (OCP) ^{150-152 439}, tubal ligation and hysterectomy ¹⁸² reduce the risk of ovarian cancer and are protective factors ¹⁸²⁻¹⁸⁵. Other

factors such as lactation, age at menarche, and age of menopause seem to have a weaker effects on risk reduction ^{150 152 185 439}. The major (monogenetic) risk factors for breast and ovarian cancer are reproductive and dietary factors.

Pakistani women have a low frequency of these traditional risk factors (Bhurgri Y 2000) the female population, on average, has high levels of fertility, early age at first pregnancy, multiple births, and prolonged breast-feeding. Although the age at first marriage has been gradually rising, women in consanguineous unions marry at earlier ages and are less likely to use modern contraceptive methods than do women from non-consanguineous marriages ⁴⁴¹. Pakistan is known to have the highest rate of consanguineous marriages worldwide, with frequencies of 60-70% being reported— ⁴⁴². Pakistani women do not tend to use exogenous hormones (OCP or hormone replacement therapy). They generally do not smoke tobacco products, although some women practice chewing pan and pan tobacco.

We know that the majority of ovarian cancer cases are sporadic. However 5-10% of cases have a hereditary basis (ie they are familial) and several genes that confer high penetrance susceptibility to ovarian cancer have been identified. Two of these, BRCA1 and BRCA2, are responsible for approximately half of all families with two or more ovarian cases in first degree relatives, and most families in which multiple cases of breast and ovarian cancer occur together ^{139 443 444}.

Within the Pakistani population, there is a paucity of the known established risk factors to explain the observed high incidence, and alternative risk factors have not been identified. It may be that the high rates of both ovarian and breast cancer in Pakistan are due to genetic factors.

It has been reported that the offspring of first cousin unions have twice the risk of developing breast and ovarian cancers than those of non-consanguineous parents and it is widely accepted that consanguinity and inbreeding increases the risk of diseases caused by homozygosity of deleterious alleles ^{441 442 445}.

5.1.2 Consanguinity

Consanguinity is defined as marriage between blood relatives and is commonly used to describe relationships that include up to second cousin marriages ⁴⁴⁶. At a biological level, consanguineous unions may increase the risk of the homozygous state in their offspring ⁴⁴⁶. This might be reflected in relatively higher probability of an autosomal recessive inherited disease and certain types of congenital malformations. The most common types of consanguineous unions include first cousin unions with uncle-niece unions or marriage to second cousins or distant relatives forming only a small proportion.

In certain parts of the world, the rate of consanguineous marriages (those between second cousins or closer) may exceed 50% ⁴⁴⁶. Accepted in certain cultures, consanguinity may also help proliferate the expression of disease-causing recessive alleles in the population. Evidence has also shown that level of consanguinity positively correlates to incidence of adult-onset complex diseases. In one example, Rudan and colleagues ⁴⁴⁷, showed that the genetic isolation of coastal island populations living in middle Dalmatia, Croatia is likely to be a factor in the high incidence of diseases such as cancer, heart disease, and stroke. Other investigators have linked the elevated levels of certain cancers among the Hutterites ⁴⁴⁸, Syrian Jewish community in Brooklyn, New York ⁴⁴⁹, Pakistanis ⁴⁵⁰, and Louisiana Acadians ⁴⁵¹ to high incidence of consanguinity within these groups.

Recent case studies have shown that two copies of highly penetrant colorectal cancer-causing alleles [PMS2 deletion ⁴⁵² and the MYH frameshift mutation ⁴⁵³ may be passed down by consanguineous parents to their offspring. Autozygous segments may harbour alleles (Ac and Bc), which when present in homozygous form can increase cancer risk for the individual. The simplest explanation is that these genes in the autozygous regions can either be (a) a pair of recessive mutant cancer genes (such as MYH and ATM; ⁴⁵⁴, (b) highly penetrant dominant cancer genes (such as BRCA2, MSH2, and

MSH6) whose biallelic mutations may lead to distinct cancer phenotypes ⁴⁵⁵, or (c) low-penetrance, dose-dependent cancer predisposition SNPs such as the 8q24 SNPs recently linked to colorectal cancer ^{456 457}. In addition, a large autozygous segment may contain multiple genes satisfying any of the above characteristics, with all of them effectively contributing to increased cancer predisposition.

5.2 AIMS

The aim of this study was to investigate the association of the VEGF polymorphism 1154 G/A and the risk for incident Epithelial Ovarian Cancer in a population based case control study from Pakistan. Included in this analysis were 606 subjects, including 303 controls and 303 cases who were matched with respect to age and ethnicity.

5.3 MATERIALS, METHODS AND SUBJECTS

5.3.1 Study Population

A total of 606 subjects were enrolled into the study between September 2005 to June 2007, consisting of 303 controls and 303 cases with a histologically confirmed diagnosis of epithelial ovarian cancer. These subjects were recruited from three centres in Pakistan (Shaukat Khanum Memorial Cancer Hospital in Lahore, Ziauddin University hospital and the Aga Khan University hospital , in Karachi), and were part of the ongoing Pakistan Ovarian Cancer Study. All subjects were of Pakistani origin belonging to the five main ethnic group (see table 1). All the EOC cases were histologically confirmed, and staging was performed according to the current International Federation of Gynaecology and Obstetrics (FIGO) classification.

Inclusion criteria for cases were a primary diagnosis of EOC for women under the age of 75 years of age with histopathologically confirmed EOC. Pathological diagnosis was based on the International Histological Classification of Ovarian Tumour recommended by FIGO . Controls were recruited from patient attending hospital based outpatient clinics and were matched to cases by age and geographical area and ethnicity. The inclusion criteria for controls were women known not to have a personal or family history of breast or ovarian cancer, or bilateral oophorectomy.

The study was approved by the ethical review committees of all the participating centres.

5.3.2 Questionnaire and Interview

A structured questionnaire was used to collect the requested information on reproductive, hormonal, and environmental risk factors as well as a detailed family history and the presence of consanguinity within the parents of the case subjects and controls. The questionnaire and consent was translated into Urdu and written consent was obtained from each study participant.

The questionnaire was completed in a face to face interview with the assistance of research officer. An information leaflet outlining the study was given to each participant prior to completing the questionnaire.

After appropriate and detailed counselling by a dedicated research officer a 10mls blood sample was taken from each study participant, in an EDTA sample tube for later DNA extraction. The sample was coded and anonymised.

5.3.3 Genomic DNA Extraction

A total of 10 ml of venous blood was collected in an EDTA tube for DNA extraction from white blood cells. DNA was extracted using phenol-chloroform reference protocol.

40ml of RBC lysis buffer at pH 8 (1mM Tris, 32mM sucrose, 0.5mM magnesium chloride(hexahydrate) and 10 ml of 100% triton) was added to approximately 10 ml of blood sample and centrifuged at 2500rpm for 20min. The blood was discarded, leaving behind a cell pellet. 2 ml of extraction buffer (40mM Tris, 7.63mM EDTA, 15mM sodium chloride and 3.5 mM SDS) was then added and vortexed briefly to resuspend the cell pellet. 0.5ml of sodium per chlorate (5M) was added to cell suspension followed by manual mixing and incubation at 65°C for 25 min. 2ml of chloroform was added and the solution was vortexed and centrifuged at 2500 rpm for 15 min. The upper layer was transferred to another tube, 2 volumes of isopropanol was added to precipitate DNA by gently mixing the tube. DNA was rinsed by centrifugation in 1 ml of 70% ethanol at 10,000 rpm for 15 mins. Ethanol was decanted and DNA was air dried, re-suspended in DI water and incubated at 37°C for 12 hours then placed at -20°C for storage.

5.3.4 Selection of Polymorphism in VEGF Gene, Primer Design and SNP Genotyping

Single nucleotide polymorphism (SNP) was selected in the VEGF gene (Table 5.1). This polymorphism is non coding and in the promoter region of the VEGF gene. Flanking sequence was retrieved from ENSEMBL. The tetra primers ARMS-PCR procedure ³⁶⁵ was used to genotype the SNP. The method employs four primers to amplify a larger fragment from DNA containing the SNP and amplicons representing each of the two allelic forms (Table 1). Primers were designed using web based software made accessible by Ye et al. (2001)([http:// cedar.genetics.soton.ac.uk/public_html/-primer1](http://cedar.genetics.soton.ac.uk/public_html/-primer1).

[html](#)). PCR was performed in a total volume of 10 µl containing 200-300 ng of template DNA, 1 pmol of each inner primer, 0.1 pmol of each outer primers, 200 µM dNTP, 2 mM MgCl₂, 1xbuffer and 0.5 U of Taq DNA Polymerase (Promega).

PCR conditions were: initial denaturation at 94°C for 7 minutes followed by 34 amplification cycles each consisting of denaturation at 94°C for 45 seconds annealing at 66.2°C for 45 seconds and extension at 72°C for 45 seconds and final extension at 72°C for 7 min. The amplified products were electrophoresed in a 2.5 % agarose gels stained with ethidium bromide. Positive and negative controls were included on each run. Agarose gel was evaluated under ultraviolet light and polymorphisms were classified as AA, AG and GG.

The genotyping results were after the TETRA ARMS PCR were read by two independent individuals. About 10% of the PCR assays were randomly repeated and the results checked for concordance.

Table 5.1: SNP ID and Primer Sequences.

refSNP ID	Polymorphism	Primer Sequence(5'-3')	Amplicon size (bp)
rs	1154 G/A	Forward inner primer(A allele): GATTTTGGAAGGACTTGCCTGATGCA	208(A allele)
		Reverse inner primer(G allele): CTGTAATGCCACTCTTTGGAGCTGCC	144(G allele)
		Forward outer primer: GTGTGATCTCTGGAATGAAAACAGGCCT Reverse outer primer: ATCAGGGTACTCCTGGAAGATGTCCACC	300(from two outer primers)

5.3.5 Statistical/Data analysis

The association between *VEGF* rs 1570360 and EOC was modelled through multivariate logistic regression analysis. Odd ratios and confidence intervals

were used to assess the occurrence of *VEGF* -1154G>A genotypes in patients with EOC cases compared with the control group. Significance testing was carried out by combining chi-square tests and then comparing the two independent proportions. Adjusted OR's for the epidemiological covariant such as history of gynaecological cancer and/or other cancer types in the family, tumour stage and grade, age and ethnicity, were determined by using a multivariate logistic regression method. Survival associated with each of the three genotypes was estimated by Kaplan-Meier survival analysis. We considered results with $P < 0.05$ as statistically significant. The statistical software package STATA 11.0 was used to carry out the statistical analyses.

The measure of Hardy-Weinberg disequilibrium was calculated as the function of the difference between the observed and expected genotype frequencies. Estimation of departure from Hardy-Weinberg proportions was evaluated with the help of D_A statistic and tests of statistical significance were applied as reported by ³⁶⁷.

5.4 RESULTS

The selected characteristics of the 303 cases and 303 controls are summarised in Table 5.2. The cases and controls appeared to be adequately matched on age and ethnicity as suggested by chi squared tests.

The results of the genotype frequencies obtained are shown in Table 5.3. The genotype distributions and allele frequencies of the *VEGF* 1154 G/A polymorphism in patients were significantly different from those of the controls. The frequency of the G allele in patients (89.4%) was significantly higher than that in the controls (84.7%) ($p = 0.013$). Genotype frequency distribution in the two groups (controls and cases) occurred in Hardy-Weinberg proportions ($P > 0.1$ in cases and controls).

Variable		number or mean (SD)
Total number of patients enrolled		296
Age at first diagnosis		43.98
FIGO Stage	I	2
	II	19
	III	155
	IV	120
Tumour grade	I	17
	II	38
	III	241
Histological subtype	serous	222
	mucinous	44
	endometrioid	21
	clear cell	9

Table 5.2 Patient characteristics

The frequencies of the G/G, G/A and A/A genotypes were significantly different from those in the controls ($p=0.037$) (Table 5.3). Compared with the A/A +G/A genotype the G/G genotype could significantly increase the risk of EOC development (OR 1.64; 95% CI; 1.12-2.39) (Table 5.3). Carriage of the A/A versus the G/G genotype may increase the susceptibility to EOC.

No significant associations between the carriage of SNP VEGF 1154G/G and the clinicopathologic variables FIGO stage, tumour grade, and age of patients at diagnosis were ascertained.

Table 5.3. Genotype and Allele distribution of VEGF 1154 G/A Polymorphism in cases and controls and association with the risk of developing EOC.

<i>VEGF 1154A>G</i> dimorphism (rs 1570360)	Controls n (%)	Cases n (%)	OR P
<i>Genotypes</i>			
<i>G/A + G/G</i>	86 (28.4)	59(19/5)	1.00
GG	217 (71.6)	244(80.5)	1.64 (1.12-2.39)
AG	79 (26.1)	54(17.8)	0.037*
AA	7 (2.3)	5(1.7)	
<i>Allele frequencies</i>			
G	513 (84.7)	542 (89.4)	0.013
A	93 (15.3)	64 (10.6)	

* P value was estimated

using chi squared test for the G/G, G/A and A/A genotype of VEGF 1154G/A SNP.

In a univariate analysis, FIGO stage, tumour grade, age of patients at diagnosis, and the carriage of all three genotypes VEGF 1154 AA, AG and GG, were associated with overall survival (Figure 5.1). Analysis indicated that the GG genotypes are associated with median survival of 15 weeks, compared to 60 weeks for the AA and AG genotypes (log rank test $p < 0.0001$). In a multivariate Cox regression model, these results remain unchanged. No interactions were found between the carriage of the VEGF 1154 G/G genotype and FIGO stage, tumour grade, or age of the patient at

diagnosis could be statistically verified ($p=0.9$, $p=0.9$ and $p=0.7$ respectively). Therefore the effect of carriage of the VEGF 1154 G/G genotype found in multiple Cox regression analysis is independent of the level of a patient's FIGO stage, tumour grade, or age at diagnosis. No significant violations of the proportional hazards assumption could be detected for any of the variables entering multivariate Cox model.

5.5 Discussion

In this population-based case-control study from Pakistan, we investigated the association of a polymorphism in the VEGF gene; VEGF 1154 A/G, to assess whether carriage of the polymorphism increases susceptibility to EOC in a population based case control study from Pakistan. We also investigated the association of VEGF 1154A/G polymorphism with the risk of development of EOC and its effects on overall survival. We decided to study this particular polymorphism, as it has been shown in other studies, to be associated with higher VEGF production, whereas VEGF 1154 A/A is associated with low VEGF production^{352 353 458}.

Our results illustrated a significant difference between the cases and controls in allele frequencies $p<0.013$ and carriage of the 1154 G allele was associated with an increased risk of ovarian cancer. This result may be explained, and is biologically plausible, as the VEGF 1154 G/G polymorphism is known to be associated with increased VEGF production.

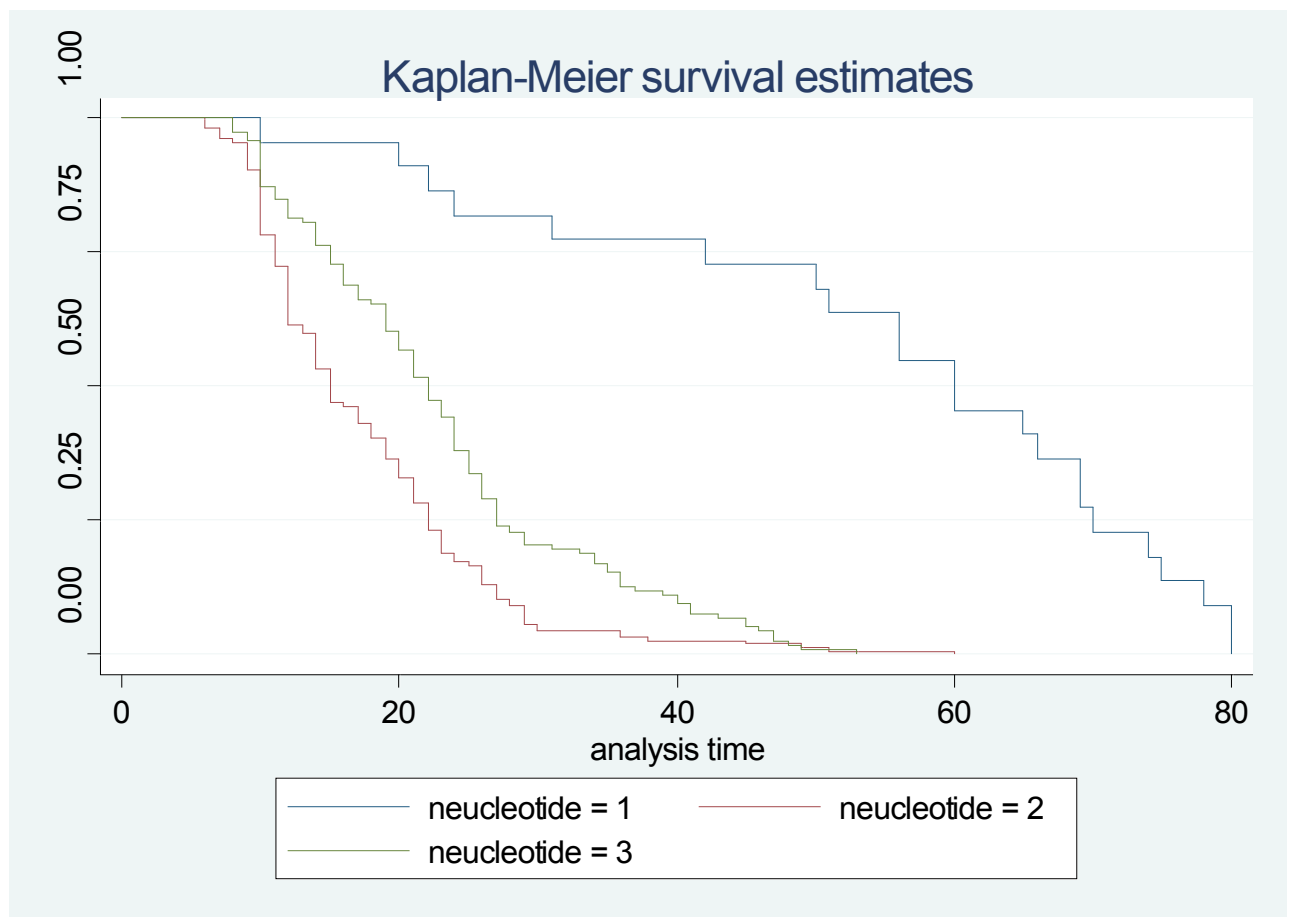


Figure 5.1 Kaplan Meier curves including the 95% confidence bands on overall survival of patients with ovarian cancer. Carriage of three genotypes AA= nucleotide 1; AG = nucleotide 2 and GG = nucleotide 3 was correlated with overall survival.

Angiogenesis the formation of new blood vessels from endothelial precursors is a prerequisite for the growth and progression of solid malignancies ¹⁰⁴. VEGF is believed to be important for angiogenesis initiation ^{25 286}. Functional polymorphisms of VEGF that regulate gene expression may contribute to the susceptibility to cancers. In ovarian cancer, in vitro studies showed that VEGF is critically important in the various steps of ovarian carcinogenesis ^{286 298}. VEGF was shown to be associated with the promotion of angiogenesis in early stage ovarian cancer, suggesting that VEGF- driven angiogenesis might be an early event in ovarian carcinogenesis ²⁹³. Over-expression of VEGF

detected immunohistochemically and elevated serum levels of VEGF have been shown to confer a worse prognosis in ovarian cancer^{459 460}.

Functional polymorphisms of VEGF that regulate gene expression may contribute to the susceptibility. The impact of these functional SNPs of the VEGF gene on tumour development has been studied using molecular epidemiological studies of several cancer types, including Prostate³⁵¹; Lung, breast⁴⁶¹ and gastric cancers.

Konac et al did not show any association between VEGF 460 C/T and 936 C/T polymorphisms and the development of gynaecological cancers including ovarian cancer. Poltauer et al, investigated three VEGF polymorphisms, VEGF 405 G/C VEGF 460 C/T and VEGF 936 C/T and prognostic parameters in ovarian cancer. None of these VEGF genotypes and haplotypes showed any significant association with patient's prognosis^{462 463}. In contrast to this study and in agreement with our results Hefner et al, in a study investigating VEGF polymorphisms and prognosis in EOC, showed that the simultaneous carriage of the three homozygous genotypes VEGF 634 C/C; VEGF 1154 G/G and VEGF 2578 C/C was associated with a shortened overall survival²⁸⁰, but none of these genotypes alone showed any statistical significance.

As the studied SNP VEGF 1154 G/G is associated with increased VEGF production therefore it can be reasonably speculated that the carriage of this genotype would lead to higher circulating VEGF levels. Furthermore our data illustrated that the carriage of the 1154 G/G SNP not only confers increased susceptibility to the development of EOC, but also the GG genotype is associated with survival estimates of 15 weeks compared to 60 weeks for AA or AG genotypes ($p < 0.0001$), this is independent of the stage of tumour, histological grade of tumour. Furthermore we have shown that in the case control study, the carriage of VEGF 1154 G/G, which is a functional polymorphism and is known to be associated with increased VEGF production, leads to increased susceptibility to EOC, in addition, carriage of the 1154 G/G polymorphism, was found to be significantly associated with

poor prognosis in patients with EOC, independent of known established prognostic factors for EOC.

Interestingly in this study we found that both cases and controls had increased proportion of homozygosity than would be expected from outbred populations. This may reflect and support the high rates of consanguinity known to exist within the Pakistani population, with rates reported of 60-70%⁴⁶⁴.

The presence of these germ line homozygous segments may be explained by consanguinity somewhere in the individual's ancestry. After all 1/16 and 1/64 of a child's genome is expected to be identical by descent if his/her parents are first and second cousins respectively⁴⁶⁵. Individuals are frequently observed to have long segments of uninterrupted sequences of homozygous markers. These long homozygous segments arise through numerous mechanisms including consanguineous marriages, in which parent pass shared chromosomal segments also known as autozygous segments; ie the two alleles in a homozygous genotype are identical by descent (IBD). Evidence has shown that level of consanguinity positively correlates to the incidence of adult –onset complex disorders including cancer.

Humans are believed to carry over a million distinct SNPs⁴⁶⁶. Determination of SNPs is a means to study the aetiology of polygenetic disorders with complex inheritance patterns⁴⁶⁵,⁴⁶⁷ For instance they affect the process of cancer development and tumourigenesis via actions on the pathways of tumour angiogenesis. This may be achieved by enhanced or reduced transcription, altered posttranscriptional or posttranslational activity, or changes in the tertiary structure of the gene product⁴⁶⁸.

Several limitations in our study need to be addressed. First the sample size of the current study may not be large enough to detect small effects from low penetrance genes, and a much larger sample size is needed to validate our finding, and to investigate the functional relevance of this polymorphism in ovarian cancer development and progression. Secondly the 1154

polymorphism investigated in our study, based on its functional consideration, may not give a comprehensive view about genetic variability in VEGF.

Due to the high level of consanguinity we observed large numbers of homozygous genotypes are present within our study, this may not be represented in other populations and may explain the high rates of ovarian cancer seen in the Pakistani population.

In the future our results should be further validated in gene expression studies and the linkage of these results to prognosis and response to platinum based chemotherapy would give us further insight into the functionality and role of the VEGF in ovarian cancer, and also identify individuals that are at increased risk of increased VEGF production. The clinical and prognostic value of this information in light of the new developments in the field of VEGF inhibitors such as Bevacizumab may prove a clinically useful tool.

Chapter 6. General Discussion

“For every increment of tumour growth there is an increment of vascular growth” Judah Folkman 1971.

In this thesis I have presented data resulting from a series of experiments and observations that illustrate the importance of angiogenesis for tumour growth and prognosis in epithelial ovarian cancer.

It is widely recognised that tumour angiogenesis is critical for tumour growth beyond 2mm^{1 469 470} and is associated with prognostic significance in a variety of solid malignancies such as breast, prostate, gastric and non small cell lung cancer;¹. However results from studies in EOC are conflicting and the clinicopathological significance has been debated^{129 316 373}. Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein expressed in a wide variety of normal and transformed cell types⁵⁵. It is a key angiogenic factor and acts as a highly specific mitogen promoting endothelial cell migration and inhibiting apoptosis¹². Studies have suggested a specific role for VEGF in various phases of ovarian carcinogenesis with effects on tumour and neovascularisation illustrated in animal models and in humans. It has been shown to have prognostic value in predicting for metastases and overall survival in various solid tumours^{298 343}.

6.1 VEGF as an Independent Prognostic Factor in Epithelial Ovarian Cancer

My results indicate that in patients with epithelial ovarian cancer, tumours that express high levels of VEGF have a worse overall survival rate compared to those with low intratumoural VEGF expression. A median survival advantage of approximately 25 months is seen with patients who have low VEGF expression ($p < 0.0001$). This result is further substantiated when a multivariate Cox regression model was constructed in which established prognostic factors such as stage of tumour, grade of tumour and residual volume of disease after primary surgery, were accounted for. The expression of intratumoural VEGF maintained statistical significance with regards to patient survival ($p < 0.0001$). My results conclude that the immunohistochemical

assessment of VEGF expression within a tumour can display further information about tumour behaviour and subsequent prognosis for the patient.

The literature to date has been unclear as to the role of VEGF expression within ovarian tumours. Some studies have shown it is a significant independent prognostic factor and have supported the results that I have presented in this thesis. Paley et al found that patients with early stage disease FIGO stages I and II, showed poorer prognosis with increased VEGF expression within the tumour³⁰¹. Shen et al showed that survival of patients with high VEGF expression was significantly worse than those with low VEGF expression and in multivariate analysis VEGF expression together with stage was an independent prognostic indicator for overall survival³⁸⁰. This was in agreement with our data. I have demonstrated that there was no association between VEGF expression and any of the clinicopathological variables including stage, and grade of the tumour, other studies have also illustrated this^{55 289 293 382}. Some studies have suggested that stage and grade are associated with VEGF expression, although these studies have a larger proportion of early stage disease³⁸⁰. High VEGF levels in the serum have also been independently associated with shorter progression free survival, and have been shown to be a prognostic factor in EOC. Prechemotherapy serum levels of CD105, transforming growth factor beta2, and vascular endothelial growth factor are associated with prognosis in patients with advanced epithelial ovarian cancer treated with cytoreductive surgery and platinum-based chemotherapy⁴⁷¹.

Similarly VEGF levels in ascitic fluid have been shown to be a prognostic factor in EOC. Mesiano et al have shown higher levels of VEGF in the ascitic fluid of patients with high stage disease.ref 289 In addition Barton et al reported an increased concentration in the serum and ascitic fluid in patients with high stage disease.295 A possible limitation in our study is that we did not correlate the levels of VEGF in the serum or ascitic fluid of patients to examine its role as a prognostic factor in EOC.

There were are a number of technical limitations of our study, and the results may have been subject to bias due to these technical limitations. As it is difficult to standardise IHC techniques, the expression quantification of the VEGF protein is subject to observer error, and the heterogeneous expression of VEGF, causes difficulties in maintaining a standardised scoring system for the quantification and assessment of VEGF. The scoring system formulated and used in this study was devised by JC and GS, the accuracy and reproducibility of this system would need to be tested in larger prospective studies. I hope that further work is carried out in the future to develop a more reproducible and quantitative method for detection /estimation of levels of VEGF in the tumour, such as used in the Hercep Test tm. Currently breast cancer patients are selected for treatment with Herceptin by IHC with a semi quantitative estimation of the levels of erb B2 in their tumour. The most commonly adopted method has become the Hercep Test (Genentech, Inc) Formalin fixed paraffin sections are reacted with antibody to erb-B2 and analysed according to the quickscore method⁴⁷². The staining intensity is scored as well as proportion of positive stained cells. A similar model could be developed to focus on VEGF to allow automated quantification of VEGF in order to create a clinical diagnostic tool.

Another limitation of my work was that the study was retrospective which brings clinical bias into the result, and the number of patients was small. In further work, it would be useful to ascertain if these results could be reproduced in a larger prospective study to address these issues.

I examined the role of VEGF as a marker of the angiogenic response in EOC; however it could be argued that VEGF is only one of the factors associated in the angiogenic pathway and further work should be carried out to ascertain the role of the other factors involved in the angiogenic response to further our understanding of this process. Furthermore I did not use microvessel counts (MVD) as a measure of angiogenesis, as the results of other studies, using this method as a means of assessing the degree of angiogenesis have been contradictory, in EOC. Hollingsworth et al reported that microvessel counts correlated with progression free and overall survival in advanced ovarian

cancer²⁸⁹. Univariate analysis showed that high MVD counts confer a worse prognosis for overall/progression free survival whereas a low MVD count was correlated with better survival. This however failed to show significance in multivariate analysis^{291 379}.

In summary my study illustrates that the use of VEGF IHC can be used to predict for disease progression and poor prognosis in EOC. This may allow the selection of a sub group of patients, who may have more aggressive disease, for adjuvant chemotherapy and, or upfront therapy with anti angiogenic or biological therapies.

6.2 Platinum sensitivity and VEGF

For patients with epithelial ovarian cancer (EOC) cytoreduction together with a combination of taxane and platinum is the standard of care. Despite this approximately 50% of advanced disease will relapse and moreover about 15-20% of cases with EOC will fail to respond to platinum based chemotherapy. The data presented in this thesis further go onto illustrate that in EOC, VEGF expression in the tumour as assessed by IHC, is significantly associated with platinum sensitivity, with high VEGF expression in the tumour predicting resistance to platinum based chemotherapy. Demonstration of over expression of VEGF in patients by IHC, not only provides the basis for a biomarker assay for assessing disease progression, but also identifies those patients that are likely to be platinum resistant and who may benefit from the addition of anti angiogenic therapy to their therapeutic regimen.

Other investigators, have reported that the elevated expression of VEGF in tumours of patients with EOC is associated with poor prognosis^{39 275 301 304 39 304}, the data presented in this thesis and the results of our study are one of the first to show that the pre-treatment intratumoural expression of VEGF is a predictor of response to platinum based chemotherapy.

Data from other groups support these findings; Bamias et al showed that high VEGF levels in the ascites of patients with advanced epithelial ovarian cancer were of poor prognostic significance. This group also reported an association of VEGF expression, within the ascites of patients with platinum resistance

433.

One possible biologically plausible mechanism for the prognostic capacity of high tumour VEGF expression may be related to its function as a regulator of angiogenesis. However the exact pathway that is involved in causing platinum resistance, in association with high VEGF levels remains unclear and it is hypothesised that other factors in the angiogenic pathway would be involved. Future work in this area could explore these factors and ascertain their role in platinum resistance.

Recent interest has focussed on the use of anti angiogenic drugs in an attempt to inhibit the pro tumour effects of VEGF. Evidence that these targeted therapies were biologically active came from the studies in 2004, using Bevacizumab (Avastin) Genentech Inc San Francisco US). Avastin, a monoclonal antibody to VEGF A, was the first agent to target angiogenesis. It has been shown to improve survival in patients with advanced disease alone, and in combination with existing chemotherapy regimens. More recently smaller molecular weight Tyrosine Kinase Inhibitors (TKIs) have also shown the ability to interfere with the VEGF signalling network.

Further work should be carried out in the future to validate the results from our study in a larger prospective study. In the future it may be possible to detect recurrence of cancer by these and other Biomarkers, within the genetic profile of patients, to predict groups of patients more at risk of having more aggressive disease, and likely to be resistant to platinum based chemotherapy. This will enable us to stratify patients, and individualise patient treatment. It may allow us to guide anti angiogenic therapy as upfront therapy, in patients who are likely to be resistant to platinum based chemotherapy

6.3 VEGF Polymorphism 1154 A/G and the risk of EOC

To further explore the role of VEGF in EOC. I studied the association of a polymorphism in the VEGF gene; VEGF 1154 A/G, to assess whether carriage of the polymorphism increases susceptibility to EOC in a population based case control study from Pakistan. I also investigated the association of VEGF 1154A/G polymorphism with the risk of development of EOC and its effects on overall survival. We decided to study this particular polymorphism, as it has been shown in other studies, to be associated with higher VEGF production, whereas VEGF 1154 A/A is associated with low VEGF production.

352-354 458

My results illustrated a significant difference between the cases and controls in allele frequencies $p < 0.013$ and carriage of the 1154 G allele was associated with an increased risk of ovarian cancer. This result may be explained and is biologically plausible, as the VEGF 1154 G/G polymorphism is known to be associated with increased VEGF production.

Functional polymorphisms of VEGF that regulate gene expression may contribute to the susceptibility. The impact of these functional SNPs of the VEGF gene on tumour development has been studied using molecular epidemiological studies of several cancer types, including prostate³⁵¹ lung , breast⁴⁶¹ and gastric cancers^{473 474}. The results however remain inconsistent.

Kataoka et al conducted a population based case control study of 1093 breast cancer cases and 1093 controls in Chinese women and found that VEGF 936 C/T was associated with a reduced risk of breast cancer in premenopausal women (Kataoka, Cai et al. 2006). In contrast another large case control study of 1489 women with breast cancer (565 women familial breast cancer from Poland and Germany and 924 unselected breast cancer cases from Sweden) showed no difference in the allele or genotype frequencies of VEGF 935 C/T between the cases and controls ⁴⁷⁵.

Konac et al did not show any association between VEGF 460 C/T and 936 C/T polymorphisms and the development of gynaecological cancers including ovarian cancer. Poltaurer et al, investigated three VEGF polymorphisms, VEGF 405 G/C VEGF 460 C/T and VEGF 936 C/T and prognostic parameters in ovarian cancer. None of these VEGF genotypes and haplotypes showed any significant association with patient's prognosis; ^{462, 463}. In contrast to this study and in agreement with our results Hefner et al, in a study investigating VEGF polymorphisms and prognosis in EOC, showed that the simultaneous carriage of the three homozygous genotypes VEGF 634 C/C; VEGF 1154 G/G and VEGF 2578 C/C was associated with a shortened overall survival²⁸⁰, but none of these genotypes alone showed any statistical significance. Furthermore in agreement with our findings, Steffenson et al Gynae Oncol 117(1); 109-16, illustrated in his work that VEGF serum levels were significantly higher in carriers of the 2578C, 460T and 405C polymorphisms of the VEGF gene, these polymorphisms are known to have functional activity and thus affect the production of VEGF protein. However, unlike our study no clear association was observed between individual VEGF genotypes and overall survival but in haplotype analysis, it was found that patients with the AGCGC haplotype,(associated with reduced VEGF production), in multivariate analysis had longer progression free survival, compared to other haplotypes.

As the studied SNP VEGF 1154 G/G is associated with increased VEGF production therefore it can be reasonably speculated that the carriage of this genotype would lead to higher circulating VEGF levels. Furthermore our data illustrated that the carriage of the 1154 G/G SNP not only confers increased susceptibility to the development of EOC, but also the GG genotype is associated with median survival estimates of 15 weeks compared to 60 weeks for AA or AG genotypes ($p < 0.0001$), this is independent of the stage of tumour, grade of tumour and residual disease status. In conclusion these data further substantiate our findings that estimation of VEGF is a prognostic factor in EOC. We have illustrated this through a series of experiments looking both at protein expression and also at a genetic level. Both sets of results are in concordance with each other. Furthermore we have shown that in the case

control study, the carriage of VEGF 1154 G/G, which is a functional polymorphism and is known to be associated with increased VEGF production, leads to increased susceptibility to EOC, in this population. In addition, carriage of the 1154 G/G polymorphism, was found to be significantly associated with poor prognosis in patients with EOC, independent of known established prognostic factors for EOC.

6.4 Study Design and Sample size

There were limitations to this study. We used a hospital based case control study, which is an appropriate strategy to easily get participants' agreement to provide tissue samples. All the study samples were obtained from hospital sources including healthy controls, the controls represented all the geographical areas of Pakistan and all ethnic groups were included. As the controls were selected from a population sample this could potentially present higher rates of participation refusal, thus inducing selection bias. In order to have an adequately powered study to test the effect of gene polymorphisms, calculations of sample sizes depend upon population prevalence, as well as the size of the effect to be estimated. Case-control studies are known for large variations, inconclusive results and lack of biological plausibility. These are usually due to small sample sizes, lack of power to show statistical significance, and control selection ^{352 476}.

Additional problems are faced in gene polymorphisms studies in cancer. Some risk factors are unknown or resources are not available to measure the known risk factors that could influence the development of cancer and access to entire statistical population of cases is not possible. In this study we did not have access to the complete demographic data of the control population. In calculating sample size if it is known that a given allele increases susceptibility for a specific cancer and its prevalence in the population is known e.g. 15%, a case-control study will need around 200 cases and similar number of controls to estimate the effect of that allele. However, if it is unknown whether the gene

causes susceptibility, as it was in our study, additional information is required for sample size calculation. This includes a biologically relevant effect size, Type 1 error (usually 0.05) and the required power of the study (usually 80% or more). Moreover, testing interactions between genes and environmental factors requires studies with additional participants ³³¹.

The study of SNP markers is an indirect association study because the susceptibility gene is not known. The power of indirect association studies is not well understood ⁴⁷⁷. The power of indirect association studies in case-control design is reduced compared with direct susceptibility gene studies. Moreover, the greater the number of independent SNP markers, the more difficult it is to achieve a significance level because of Bonferroni correction. In this study the VEGF 1154 A/G SNP was found to be in complete linkage. Moreover, we had hypothesized that the VEGF gene considered in this study is a candidate susceptibility gene because of the functional role of their polymorphisms in angiogenesis and carcinogenesis as suggested by other studies ³⁵². Hence the power of the study was not reduced significantly.

This study had only one genetic polymorphism and this has different prevalence's in different ethnic groups. We had chosen that particular SNP based on the known important role of VEGF driven angiogenesis in ovarian carcinogenesis and based on previously published data on the role of VEGF polymorphisms in other malignancies ⁴⁷⁸⁻⁴⁸⁰.

It was difficult to predict a biological effect on size because it was not known to what extent does a given SNP increases the susceptibility to ovarian cancer. To achieve an acceptable sample size, an effect size of 20% was assumed to be biologically relevant and other values mentioned previously were included in calculating sample size, as described in the Methods and Materials section. Since a number of variables, described previously, were to be used in the analysis, all efforts were made to recruit as many study subjects as possible to maintain an acceptable power for subgroup analysis irrespective of the initial sample size calculation.

The study was carried out exclusively on a population of Pakistani ancestry. In this study we found that both cases and controls had an increased proportion of homozygosity, than would be expected from outbred populations. This may reflect and support the high rates of consanguinity known to exist within the Pakistani population, with rates reported of 60-70%⁴⁴². We decided to study the Pakistani population due to the presence of high rates of consanguinity in its population, as several studies link consanguinity to cancer. They suggest that autozygosity (a genomic consequence of consanguinity) may be factor in cancer predisposition, there are clear correlations between the incidence of cancer and degrees of inbreeding on a number of population based studies ^{450 481}.

Individuals from consanguineous unions frequently have been observed to have long segments of interrupted sequences of homozygous markers, after all 1/16 and 1/64 of a child's genome is expected to be identical by descent if his or her parents are first cousins and second cousins respectively; ⁴⁶⁵. These long segments arise by various mechanism including consanguineous marriages, in which parents pass shared chromosomal segments also known as autozygous segments, the two alleles in a homozygous genotype are identical by descent (IBD)⁴⁸². Evidence has shown that the level of consanguinity positively correlates to the incidence of adult onset complex disorders. The fact that I studied the Pakistani population may also be viewed as a limitation of our study as the homozygous gene pool may be postulated to be greater than would be expected in other populations. To show an association you would need a smaller number of cases and controls, as expected genotype frequencies in individuals from different genetic background vary raising the problem of genetic background. It is difficult to compare genotype frequencies derived from our study in patients with ovarian cancer to other populations, and a multiethnic trial would be needed to further validate the effect we have observed.

Age, stage and grade of disease and residual disease status, did not affect the distribution of genotypes in the control and the case populations. Association of a SNP with the dependent variable does not imply a causal relationship, so the confounding effect of the known and unknown variables had minimal impact on the interpretation of the findings. An important purpose of this study was to evaluate the role of VEGF 1154 G/A as a potential biomarker of ovarian cancer. The sensitivity and specificity of the biomarker would be validated by future studies on a much larger sample sizes and over a longer period of time.

6.5 Future Work and Clinical Relevance

Over the last several decades, oncology research and cancer treatment have concentrated primarily on the cancer cells. Unfortunately, despite the intensive quest to find new and more effective compounds for chemotherapy, the survival rate of patients has not significantly changed. In 1971 Judah Folkman proposed that a solid tumor cannot grow without inducing angiogenesis. Target based therapies, are emerging as the new frontier in solid tumour oncology. Extensive research has been invested in identifying novel therapeutic targets and biological targets that might enhance the therapeutic index in treating ovarian cancer. Recent interest has focussed on the use of antiangiogenic drugs in an attempt to inhibit the pro-tumour effects of VEGF. Evidence that these targeted therapeutics were biologically active came from studies in 2004, using bevacizumab (Avastin®) - Genentech Inc. San Francisco, US) and was the first agent to target angiogenesis. It was licensed as a recombinant humanised monoclonal antibody (administered three-weekly) for the treatment of advanced solid cancers, and its mode of action is by targeting the isoforms of VEGF, which circulate in the bloodstream, preventing the ligand binding to its cognate receptor. Avastin® has been shown to improve survival in patients with advanced disease alone, and in combination with existing chemotherapy. More recently, smaller

molecular weight tyrosine kinase inhibitors (TKIs) have also shown the ability to interfere within the VEGF signaling network. This class of agents (aminophthalazines) includes Vatalinib (PTK787/ZK222584, Novartis, Basel, Switzerland) which acts by targeting VEGFR-1, -2 and -3, PDGFR- β and c-Kit, and has shown to improve progression-free survival of colorectal cancer patients when administered in combination with FOLFOX in Phase III trials; ⁽⁴²⁹⁾. Anti-VEGF therapy causes a slowing of tumour progression, resolution of malignant effusions and synergy with cytotoxic agents. It is also being studied as a first line treatment in a US trial (GOG 218) and a European Trial (ICON 7) in EOC.

Currently, there are many ongoing efforts to develop biological markers that predict sensitivity/resistance to particular strategies in EOC. The clinical relevance and pertinence of this study shows that measurement of the level of VEGF, determined by IHC in archival material, might be used for selection of patients likely to benefit from the addition of anti-VEGF therapies to front line platinum/ paclitaxel chemotherapy, and involve the development of a diagnostic clinical test. In the future it may be possible to detect recurrence of cancer by these and other Biomarkers, within the genetic profile of patients, to predict groups of patients likely to have more aggressive disease, and likely to be resistant to platinum based chemotherapy. This will enable us to stratify patients, and individualise patient care. It will allow us to guide anti angiogenic therapy as upfront therapy, in patients who are likely to be resistant to platinum based chemotherapy.

The present study shows that the SNP in the VEGF gene 1154 G/A is associated with increased susceptibility and poor prognosis in patients with ovarian cancer. These results would need to be validated in larger, prospective gene expression studies. Future work could examine other functional polymorphisms in the VEGF gene as well as other polymorphisms in candidate genes in the angiogenic pathway to establish a combination of genotypes that may serve as markers for susceptibility to EOC, prognosis, and drug response in EOC, This will, we hope improve the prognosis of patients with this disease.

Chapter 7. REFERENCES

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